EXTRACTION OF CYSTATIN (ENZYME INHIBITORS) FROM ANIMAL SLAUGHTERHOUSE WASTES

by

Harish Namasivayam Somasundaram

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DEDICATED

TO

MY FAMILY

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ABSTRACT

The ammonium sulphate precipitation method was used for the extraction of cystatin from bovine lungs, pancreas and a mixture of lungs and pancreas. The main aim of this study was to determine the highest cystatin yield from each of the samples and make recommendations to the industry as to whether a separation of these animal wastes are needed during the slaughtering and rendering processes. The effects of incubation time (15, 30, 45, 60 mins), pH (6.5, 7.0, 7.5, 8.0) and ammonium sulphate saturations (25-45% and 45-65%) on total protein (TP), total activity (TA), specific activity (SA) and yield (Y) were studied. The optimum conditions for total activity (TA), specific activity (SA) and yield (Y) of cystatin during ammonium sulphate extraction were an incubation time of 30 min, a pH of 7.5 and an ammonium sulphate saturation of 45-65%. Among the three samples, the lung produced the high yield of 60.07% at optimum conditions. Under these optimum conditions, the bovine pancreas and the mixture of lung and pancreas produced 39.57% and 51.89%, respectively. The effects of pH and temperature on the maximal inhibitory activity of cystatin obtained from the three samples were evaluated. The optimal pH and temperature for all the three samples were 7.5 and 50 ºC, respectively. The bovine lung sample exhibited a maximal inhibitory activity of 56.8 % at the optimal pH of 7.5 and temperature of 50ºC. Under the same conditions of pH and temperature, the bovine pancreas and the mixture of lung and pancreas samples showed an inhibitory activity of 25.8% and 43.8%, respectively. The bovine lung sample produced the highest values of TP, TA, SA, and Y of cystatin. During the slaughtering process, the lung should be separated from the pancreas in order to recover better cystatin yield.

LIST OF ABBREIVATIONS AND SYMBOLS USED

BSA:	Bovine Serum Albumin
DEAE:	Diethylaminoethyl
ES:	Enzyme-Substrate Complex
Cp:	Protein Concentration
CPI :	Cysteine Protease Inihibitor
DEAE:	Diethylaminoethyl
E:	Enzyme
E:	Enzyme
EDTA:	Ethylenediaminetetraacetic Acid
I_A:	Activity of Enzyme
IA:	Inhibitory Activity
P:	Protein
P:	Product
PI:	Proteinase Inhibitors
S:	Substrate
SA:	Specific Activity
TCA:	Trichloroacetic acid
TP:	Total Protein
TU:	Total Units
V:	Volume
Y:	Yield

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CHAPTER 1. INTRODUCTION

The world production of cattle, sheep and goat livestock, hog and chicken livestock in 2011 were 1,465,154,678, 2,017,712,657, 967,164,630 and 58,109,569,000 heads respectively. The cattle, sheep and goat livestock, hog and chicken livestock produced in 2011 in the USA were 92,682,400, 8,476,000, 110,956,304 and 8,683,643,000 heads and in Canada were 12,155,000 heads, 909,300, 21,269,500 and 638,782,000 heads, respectively (USDA, 2012; FAO, 2012; SCAD, 2012).

Only a portion of the slaughtered animal is consumed by humans as food, the other in-edible parts are considered to be wastes. These animal wastes include the animal manure and ingested food, inedible parts of the animal and the dead animals. The average amount of body waste per animal varies with the animal type, it is 37-44% for cows, 47-55% for sheep or goat, 27-30% for pigs, 28-32% for chickens and 22% for turkeys.

The disposal of animal waste has been a difficult enviro-economic problem for a very long time. Animal excretory wastes are applied to the land as fertilizers and used for production of biogas. The wastes from slaughter houses can be managed through a process called animal rendering, in which these wastes are converted into various useful commodities including lard, beef tallow, meat and bone meal, feather meal, poultry meal and yellow grease (GAO, 2002). The animal wastes considered for the rendering process includes the wastes from animals like cattle, poultry and pigs (Prokop, 1992). The raw materials for the animal rendering process include fat, bone trimmings, lungs, pancreas, intestinal wastes, blood, feathers and inedible meat scraps of mixed species (IFC, 2007). Beef tallow can also be used in the production of biodiesel. The US tallow and lard production in 2010 were 3,688,000 and 350,000 tonnes, whereas the Canadian tallow and lard production for the same year were 262,000 and 65,118 tonnes, respectively (FAO, 2010).

Proteolytic enzymes that take part in protein catabolism by the catalytic hydrolysis of peptide bonds in their target proteins are termed proteases (Ivanov et al., 2006). Proteases are classified into four classes: aspartic proteases, cysteine proteases, metalloproteases and serine proteases (Lingaraju and Gowda, 2008). Cysteine proteases

(CPs) are involved in a number of physiological and biological processes as they play a key role in heart diseases as well as numerous other diseases. Hence, proper regulation of CPs should be maintained (Cheng et al., 2012).

The proteolytic activities of the proteinases are inhibited by the proteinase inhibitors (PIs) (Lingaraju and Gowda, 2008). The characterization and identification of various proteolytic activities of the compounds present in the extracellular matter lead to different classes of proteinase inhibitors. There are two categories of proteinases based on their spectrum of activity: non-specific and specific proteinase inhibitors (Hibbetts et al., 1999). Cystatins are the proteinases which inhibit the cysteine proteases. These proteinase inhibitors are present in microorganisms, plants, animals and humans with a wide range of applications. Cystatins present in plants act as a defense mechanism protecting the plants against harmful organisms. Cystatins in animals and mammals have various functions including: (a) blocking unnecessary proteolytic reactions by which several pathological disorders like alzheimer's disease, purulent bronchitis, atherosclerosis and aneurysm are prevented and (b) playing a key role in treating various lung disorders (Wolter and Chapman, 2000). The main focus of this study was to extract cysteine protease inhibitors (CPIs) from bovine slaughterhouse waste material specifically the lungs and pancreas.

CHAPTER 2. OBJECTIVES

The main focus of this study was to extract cystatin (cysteine protease inhibitors) from different bovine parts (lungs and pancreas). The specific objectives were:

1. To determine the optimal conditions for the extraction of cystatin from three sources (lungs, pancreas and a mixture of lungs and pancreas) using ammonium sulphate precipitation method and evaluate the effects of the following parameters on total protein content (TP), total inhibitory activity (TA), specific activity (SA) and yield (Y):
   (a) Incubation time (15, 30, 45, 60 mins)
   (b) pH (6.5, 7.0, 7.5, 8.0)
   (c) Ammonium sulphate saturation (25-45% and 45-65%)

2. To optimize the maximal inhibitor activity of the extracted samples obtained from optimized extraction conditions by evaluating the effects of the following parameters
   (a) pH (3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5 and 11.5)
   (b) Temperature (30, 40, 50, 60, 70, 80 and 90 ºC)

3. To compare the yield, specific activity, total enzyme inhibitory activity of cystatin extracted from these sources and make recommendations to the industry regarding their collection, preservation and processing.

CHAPTER 3. LITERATURE REVIEW

3.1. Livestock Production

The animals used for slaughtering include cattle, goat and sheep, hogs, poultry. Table 3.1 shows the trend for cattle, goat and sheep, pigs and poultry for the period of 2002 – 2011. As of year 2011, the world cattle livestock production for slaughtering was 295,827,648 heads, of which the USA produced about 35,108,100 heads and the Canada produced 3,390,900 heads. The world goat and sheep livestock production for slaughtering in 2011 was 946,827,694 heads of which the USA produced 2,257,500 heads and Canada produced 721,200 heads. The world production of hog livestock for slaughtering in 2011 was 1,382,927,240 heads of which USA produced 66,361,000 heads and Canada produced 21,262,200 heads. The world production of poultry for slaughter in 2011 was 20,708,002,000 heads of which USA produced 2,683,643,000 heads and Canada produced 164,600,000 heads.

3.2. Slaughtering of Livestock

Based on their product, slaughterhouses are categorized into two: simple slaughterhouse and complex slaughterhouse. Simple slaughterhouses are designed to slaughter and produce fresh meat in whole, half or quarter carcass form. Complex slaughterhouses are designed for slaughtering and also for rendering, paunch, hide and hair processing. There are also packinghouses, which produce cured, smoked, canned and other meats after slaughtering. The wastes from packinghouses are comparable with that of the simple slaughterhouses.

Slaughtering of animals after being transported to slaughter houses includes series of processes: stunning of livestock, bleeding, hide removal (cattle) or dehairing (hogs) or defeathering (poultry), chilling or freezing and rendering (FAO, 2001). The work flow of a red meat slaughterhouse is shown in Figure 3.1. The slaughterhouses in Canada are of two types: commercial and non-commercial. The commercial slaughtering takes place in the federally approved and inspected slaughterhouses. The non-commercial slaughtering

Table 3.1. Trends of livestock produced and slaughtered during the period of 2002-2011 (FAO, 2011).

Year	Cattle			Sheep			Hog			Poultry		
	World	USA	Canada	World	USA	Canada	World	USA	Canada	World	USA	Canada
	(Millions tonnes)											
2002	59.6	11.8	1.69	11.5	0.10	0.01	89.1	8.92	1.85	11.5	17.0	1.11
2003	59.9	11.7	1.31	11.8	0.09	0.01	92.6	9.05	1.88	11.8	17.2	1.11
2004	60.9	10.8	1.49	12.2	0.08	0.01	93.0	9.30	1.93	12.2	18.0	1.12
2005	62.2	10.8	1.67	12.6	0.08	0.01	95.4	9.38	1.92	12.6	18.6	1.16
2006	64.0	11.2	1.59	12.8	0.08	0.01	97.3	9.55	1.89	12.8	18.8	1.16
2007	65.7	11.1	1.61	13.2	0.08	0.01	100.2	9.95	1.89	13.2	19.3	1.20
2008	65.8	11.4	1.28	13.3	0.08	0.01	102.8	10.59	1.94	13.3	19.8	1.22
2009	66.4	11.2	1.59	13.4	0.08	0.01	104.4	10.44	1.94	13.4	19.9	1.21
2010	66.6	11.2	1.60	13.3	0.76	0.01	107.1	10.18	1.92	13.3	19.5	1.21
2011	66.3	11.3	1.60	13.4	0.69	0.01	108.6	10.33	1.95	13.4	19.7	1.22

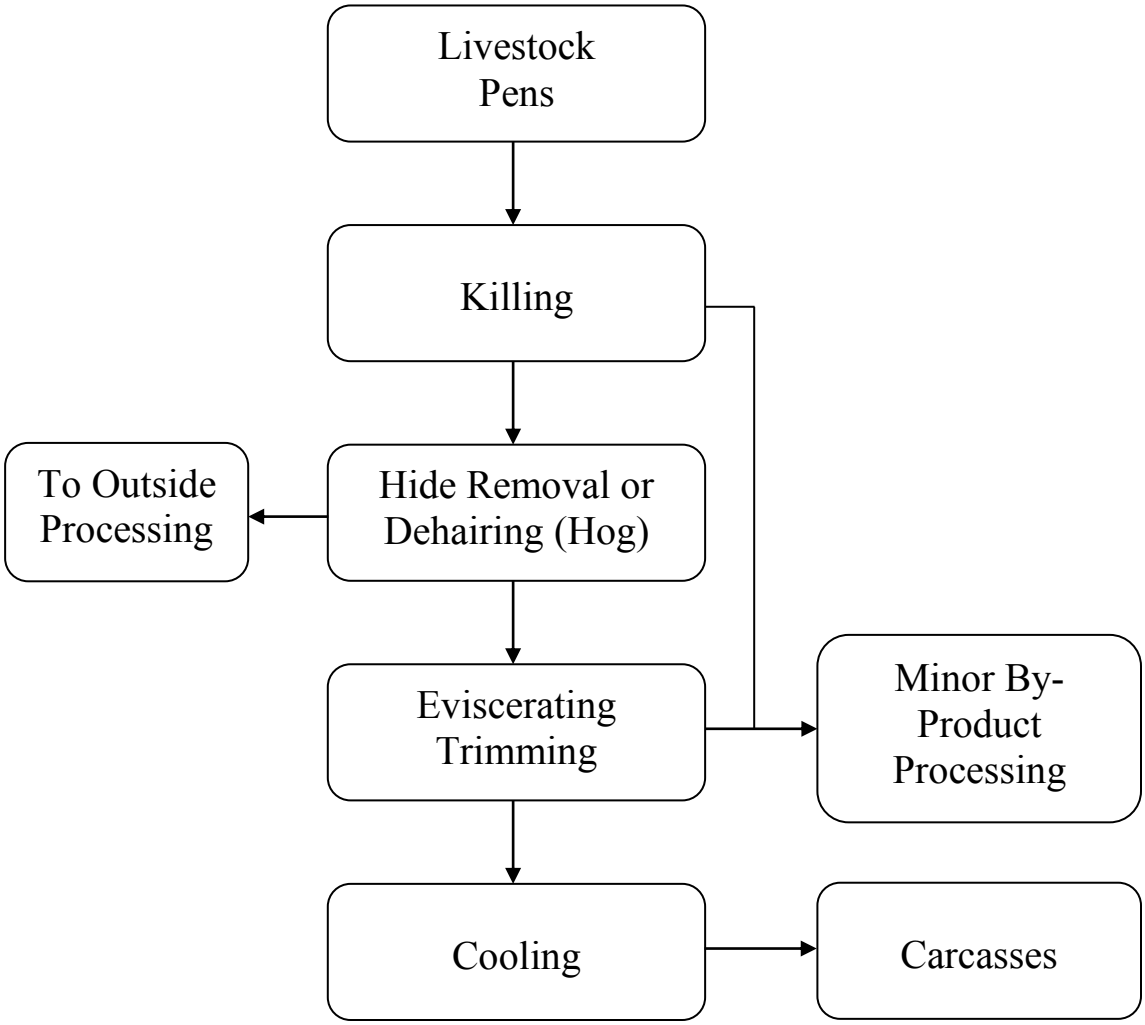


Figure 3.1. Work flow of a red meat slaughterhouse (USEPA, 1974)

takes place in the farm and it is also known as uninspected slaughtering. The non-commercial slaughtering is minimal when compared with the commercial slaughtering (SCAD, 2012).

3.2.1. Handling and Transport

Handling and transport of livestock are considered to be important before slaughtering. Excited animals have high heart rates and will be difficult to move. Isolation may cause agitation and the animals should be in crowds. The animals should be handled with care when they get transported. Transporting methods include: moving on foot, by road, by rail, on ship and by air. Negative effects of poor transport include: stress, bruising, trampling, suffocation, heart failure, heat stroke, sun burn, bloat, poisoning, predation, dehydration, exhaustion, injuries and fighting. All these effects should be avoided for better products. The driving should be smooth and without sudden stops with suitable climatic conditions (CFIA, 2013; FAO, 2001).

3.2.2. Stunning of Livestock

The process of making an animal unconscious before slaughtering, in order to eliminate pain or stress, is called stunning.. Percussion stunning, electrical stunning, and carbon dioxide gas stunning are the commonly practiced stunning types. Percussion stunning provides a physical shock to the brain of livestock. Tools of percussion stunning include: captive bolt pistol, hand-held barrel captive bolt gun and mushroom bolt gun. This type of stunning is preferred for cattle, pigs, sheep and goats as well as horses and camels. Electrical stunning produces electroplectic shock in the brain of livestock. Tongs are used to achieve this type of stunning. This type of stunning is preferred for poultry and ostriches. Different concentrations of carbon dioxide gas are used in carbon dioxide stunning of pigs and poultry (FAO, 2001).

3.2.3. Bleeding of Livestock

The bleeding process is a step in slaughtering, where the blood vessels in the neck are severed to drain blood. The bleeding is achieved by a sharp knife. Bleeding is

performed after the stunning process while the livestock is unconscious. So bleeding should be done quickly and completely (Roca, 2002).

3.2.4. Hide Removal

Following the bleeding process, the livestock will undergo hide removal (cattle), dehairing (hogs) or defeathering (poultry). The carcasses are then processed with their respective facilities available in the appropriate type of slaughterhouse. The waste products are transferred to rendering industries (FAO, 2001).

3.2.5. Rendering Process

About 30-50% of the animals raised for meat, eggs, milk and fibers are not used for human consumption and hence these raw materials are rendered into useful products by a process referred to as animal rendering (Meeker and Hamilton, 2006). The rendering process refers to the processing of natural or high fat (fat added) raw materials. The rendering process separates fats and water from tissues with the application of heat. Based on the temperature (high or low) used, there are two types of processes: the dry which uses low temperature (under 45°C) and the wet method which uses high temperature (over 45°C). The natural and additional fats (edible trimmings from cutting) are rendered by the dry method (Taylor and Woodgate, 2003). The rendering process involves a series of conveyers, presses and centrifuges and results in the separation of fats from the solid proteins. The finished fats are put in separate tanks and the proteins are pressed into a cake for feeding livestock (SCAD, 2012).

The wastes from slaughterhouses include fat trimmings, meat scraps, viscera, bone, blood and feathers (FAO, 2001). Organs like heads, lungs and intestines were transferred in the past to inedible rendering industries for the production of meat and bone meal (MBM), which was previously used as animal feed (Verheijen et al., 1996). Beef tallow and lard are the major products of the rendering industries and they have high calorific and nutritional values. The advantages of the rendering process are that most pathogens are destroyed and the waste can be recycled (Haines, 2004). However, in spite of these advantages, it is undesirable to use meat and bone meal (MBM) as animal feed due to the problem of bovine spongiform encephalopathy (BSE). BSE is commonly referred as

"mad cow disease" (Washer, 2005; Harakeh et al., 2002) as it is a neuro-degenerative disease found in cattle. It has been detected in 23 countries around the world including Canada (GAO, 2002). The first BSE case was found in Canada in May 2003. Canada has taken definitive measures for controlling the disease, such as enhanced surveillance program and feed (MBM) ban. Thus, animal feeds are no longer produced by rendering industries (CFIA, 2013).

The percentage of abdominal wastes of cattle, goat and sheep, hogs are shown in Table 3.2. The organs like heads, lungs and intestines which were initially transferred from slaughterhouses to rendering plants are considered wastes (FAO, 2001). However, the lungs and intestine are good sources of cystatins which are involved in the inhibition of cysteine proteases (Khan and Bano, 2009; Priyadarshini and Bano, 2009).

3.3. Slaughterhouse Wastes

During the slaughtering process of animals, there are certain amounts of wastes produced. The carcasses exceed 50% of the live weight kill (LWK). The carcass includes hide, skin, blood, rumen contents, bones, horns, hoofs, urinary bladder, gall bladder, uturus, rectum, udder, foetes, snout, ear, penis, meat trimmings, hide and skin trimmings, condemned meat (meat classified at inspection as unfit for human consumption), condemned carcass (meat classified at inspection as unfit for human consumption at carcass stage), oesophagus, hair and poultry offals (feathers, heads). Carcasses also include removed internal organs (eyeballs, intestine, pancreas, and lung) especially in the intestinal cavity (FAO, 2013). The average dressing weight ranges from 40-67% for cattle, 56-69% for hog, 28-30% for poultry and 40-45% for sheep and lambs (Fahmy and Davis, 1996; FAO, 2011). The weight percentage of carcass per live weight for cattle, hogs, goat and sheep, poultry is shown in Table 3.1 (SCAD, 2012).

3.4. Proteases

3.4.1. Nature and Mechanism of Proteases

Proteases cleave specific peptide bonds in their target proteins (Ivanov et al., 2006; Habib and Fazili, 2007). The catalysis of hydrolysis of the peptide bonds results in the

Table 3.2. Lung and abdominal waste composition for cattle, hog, goat and sheep (SCAD, 2012).

Type of livestock	carcass/live weight (%)	Abdominal waste (%)	lung and pancreas waste (%)
Cattle	44	7	3
Hog	30	3	1
Goat and sheep	55	5	2

primary structure of the proteins and the process is termed proteolysis (Bode and Huber, 2000). The hydrolysis action of the proteases is shown in Figure 3.2.

Proteases comprise about 2% gene of most organisms, second to transcription factors (Hedstrom, 2002; Dixon and Webb, 1979). The distribution of proteases is vast (they are found in microorganisms, plants to animals). The key functions of proteases include various biological processes and regulation of host survival. In animals, in addition to the basic functions they are also involved in processes like digestion, defense mechanisms (blood clotting), inflammation, fibrinolysis, tissue remodeling, cell cycle progression, DNA replication, immune response, ovulation and fertilization (Ivanov et al., 2006; Garcia-Carreno, 1992). Deregulation of these functions leads to numerous diseases such as cancer, rheumatoid arthritis, Alzheimer, and cataracts (Powers et al., 2002).

There are two types of proteolysis processes: limited and unlimited. Limited proteolysis governs the activities of enzymes, proteins and peptidases. Unlimited proteolysis breaks proteins into amino acid constituents (Bode and Huber, 2000). The mechanisms of proteases allow the synthesis of inactive pre-proteins and activate them. The specificity of the substrate that reacts with the enzymes has control on their activity, but it does not help in a controlled regulation of their activities. Diagrammatic representation of the synthesis of pre-proteins and the substrate control on proteases are shown in Figure 3.3. These factors lead to a need for additional control over the proteases. The protease inhibitors (PIs) aid in controlling the factors and help in the maintenance of protease activity by inhibiting them (Habib and Fazili, 2007).

3.4.2. Classification of Proteases

Proteases can be classified under three different types: activity with terminal groups, structure and catalytic mechanisms.

3.4.2.1. Terminal Groups: Based on the reaction with the terminus of a polypeptide chains, the proteases are divided into two groups: exopeptidases (peptidases) and

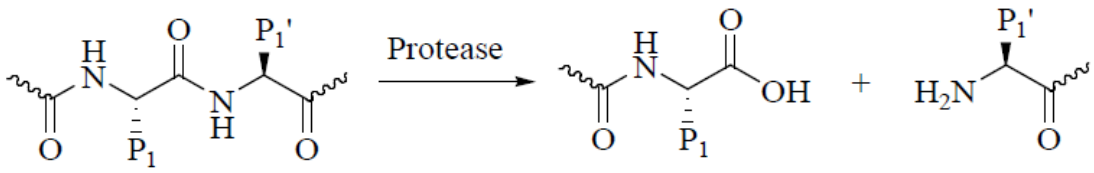
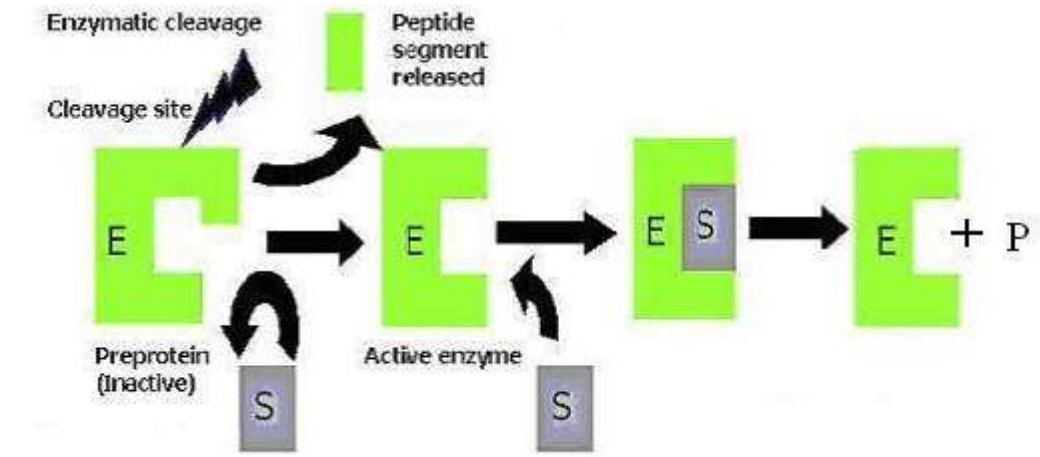
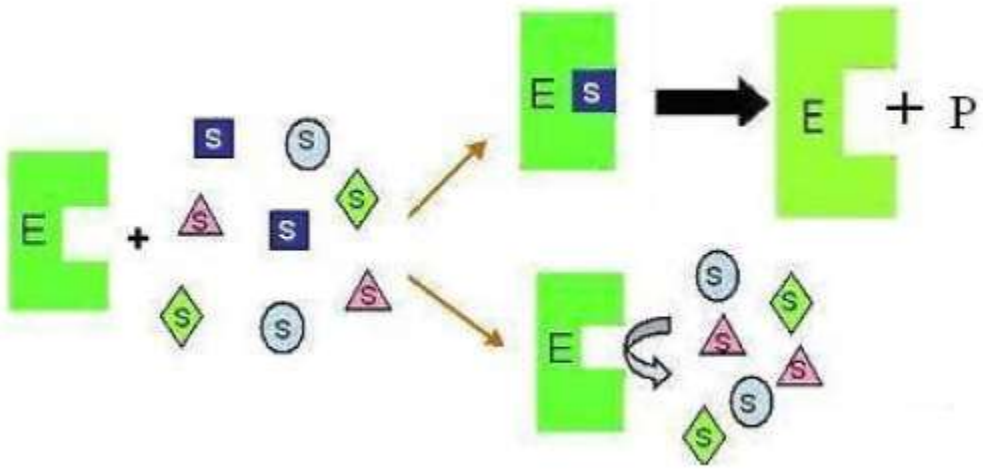


Figure 3.2. Catalytic hydrolysis of amide bonds by proteases (Voet et al., 1998)



(a) level of synthesis



(b) substrate specificity

Figure 3.3. Control of protease activity by controlling level of synthesis and substrate specificity ; E- enzyme, S- substrate and P – products (Habib and Fazili, 1998).

endopeptidases (proteinases). The exopeptidases react only in the terminal region (N-terminal or C- terminal). The endopeptidases react internally with the polypeptide chains (Beynon and Bond, 2001; Garcia-Carreno, 1992).The subclasses of exopeptidases and endopeptidases and their activities are shown in Table 3.3. The term "protease" is used for either exopeptidases or endopeptidases, whereas "proteinases" is used solely for endopeptidases (Hibbetts et al., 1999; De Leo et al., 2002). Amino peptidases, carboxypeptidases and dipeptidases belong to exopeptidases. The endopeptidases are further classified by their catalytic mechanism (Hibbetts et al., 1999).

3.4.2.2. Structure: The MEROPS database (since 1996) provides the classification of proteases based on their structure (MEROPS, 1996). This type of classification includes families and clans. The proteases with homologous peptidase units are grouped under a family. Families with identical tertiary structure form clans. A mixture of exopeptidases and endopeptidases might be found under the same family (Rawlings et al., 2012). The classification holds 180 families and 39 clans. Some important clans and families are shown in Table 3.4.

3.4.2.3. Catalytic Mechanism: Proteases are classified based on their catalytic mechanism (Hibbetts et al., 1999). The catalytic mechanism refers to the nature of the specific catalytic residue found at the active site of proteases. The classification includes major four classes; serine proteases, cysteine proteases, aspartic proteases and metalloproteases (Smith and Simons, 1985; Powers et al., 2002). Apart from the discovery of the four major classes, the threonine proteases and glutamic proteases were added to the protease classification in 1995 and 2004, respectively (Powers et al., 2002; Christensen, 2012). The classification, catalytic residue, proteins and their important functions are shown in Table 3.5.

3.4.3. Serine Proteases

Serine proteases are diverse and vastly distributed in all organisms. They are the most extensive class of proteases found in prokaryotes, eukaryotes and viruses from each other. All serine proteases have similar catalytic mechanisms and they are distinguished

Table 3.3. Exopeptidases and endopeptidases - Subclasses and their activities (Gan, 2005).

Type	Subclass	Activity
Exopeptidases	Aminopeptidases	Remove a single amino acid from free N-terminal
	Dipeptidyl peptidases	Remove a dipeptide from free N-terminal
	Tripeptidyl peptidases	Remove a tripeptide from free N-terminal
	Carboxypeptidases	Remove a amino acid from free C-terminal
	Peptidyl dipeptidases	Remove a dipeptide from free C-terminal
	Dipeptidases	Cleaves dipeptides
	Omega peptidases	Removes terminal residues that are substituted, cyclized or linked by isopeptide bonds
Endopeptidases	Oligopeptidases	Cleave preferentially on substrates smaller than proteins

Table 3.4. Structural classification of proteases (Rawlings et al., 2012).

Clan	Family	Example
PA	S1	Trypsin
	S2	Streptogrisin A
	S29	Hepatitis C virus NS3 polyprotein peptidase
	C30	Mouse hepatitis coronavirus picornain 3C-like endopeptidase
CA	C1	Papain
	C2	Calpain
	C19	Isopeptidase T
SB	SB	Substilin
CX	C15	Pyroglutamyl peptidase 1
	C26	γ -Glutamyl hydrolase
	C33	Equine arterivirus Nsp2 endopeptidase
	C40	Dipeptidyl-peptidase V
	C41	Hepatitis E cysteine proteinase
AA	A1	Pepsin
	A2	HlV 1 retropepsin
	A3	Cauliflower mosaic virus endopeptidase
	A9	Simian foamy virus polyprotein peptidase
MA	M10	Interstitial collagenase

Table 3.5. Classification based on catalytic mechanism (Garcia-Carreno, 1991; 1992; Baird et al., 2006; Schaechter, 2009; Christensen, 2012)

Protease class	Amino acid in active site	Enzymes	Origin of enzyme
Serine	Ser (195,221), Hys(57,64) and	Trypsin	Pancreas
	Asp(32,102)	Chymotrypsin	Pancreas
		Subtilisin	*Bacillius subtilis*
Cysteine	Cys(25), His (159) and Asp(158)	Papain	Papaya latex
		Chymopapain	Papaya latex
		Ficin	Ficus latex
Aspartic	Asp (33) and Asp(213)	Pepsin	Gastric juice
		Chymosin	Gastric juice (young animals)
		Cathepsin D	Liver, spleen
Metallo	Zn, Glu(143,270) His(213) and	Thermolysin	*Bacillius thermoproteolyticious*
	Try (248)	Carboxypeptidase A	Bovine pancreas
Glutamic	Q(107,133) and E(190,219)	PepG1	Fungus- *scytalidium lignicolum*
Threonine	Thr (195)	Yeast proteases A,B	Proteasome - *Saccbaromyces cerevisiae*

by their clans. They also host important physiological and pathological functions (Gan, 2005; Cera, 2009).

The mechanism of serine proteases mainly depends on the formation of a catalytic triad, which has Ser 195 on one side and Asp 102 and His 57 on the other side. It also possesses a oxyanion biding site. The structure of a serine protease showing the catalytic triad and oxyanion binding site is shown in Figure 3.4. (Walker and Lynas, 2000; Hedstrom, 2002). Haq et al. (2004) state that the catalysis includes two steps: acylation (formation of acyl enzyme) and deacylation (formation of tetrahedral transition state). Serine proteases include important enzymes such as trypsin, chymotrypsin and elastase (Cera, 2009).

The important functions of serine proteases include: digestion, blood coagulation, apostatis, reproduction, wound healing, allergic conditions such as asthma and rhinitis (Walker and Lynas, 2000; Hedstrom, 2002; Gan, 2005). Serine proteases are critically associated with the immune responses (hemolymph coagulation, antimicrobial peptide synthesis and melanization) in various invertebrates (Zhu et al., 2007). Serine protease activity in cells and living organism against hepatitis C virus was studied by Wang et al. (2010). They used a mice model and discovered that serine protease activity can be used for drug discovery against hepatitis C virus. Molecular cloning, characterization and expression of serine protease in scallops were studied by Zhu et al. (2007). They proved that there is high structural and sequence similarity that could be facilitated for wound healing and immune response. Myofibril-bound serine protease was purified and characterized from lizard fish

3.4.4. Aspartic Proteases

The Aspartic proteases are considered to be a small class of proteases with 15 members. They are widely found from retroviruses to humans (Szecsi, 1992; Eder et al., 2007). They are secreted as digestive enzymes and hence found in the stomach of mammals. Aspartic proteases are also found as lysosomal proteases (cathepsin D and E), in the kidney (renin) and also in yeasts and fungi (rhizopuspepsin, penicilliopepsin)

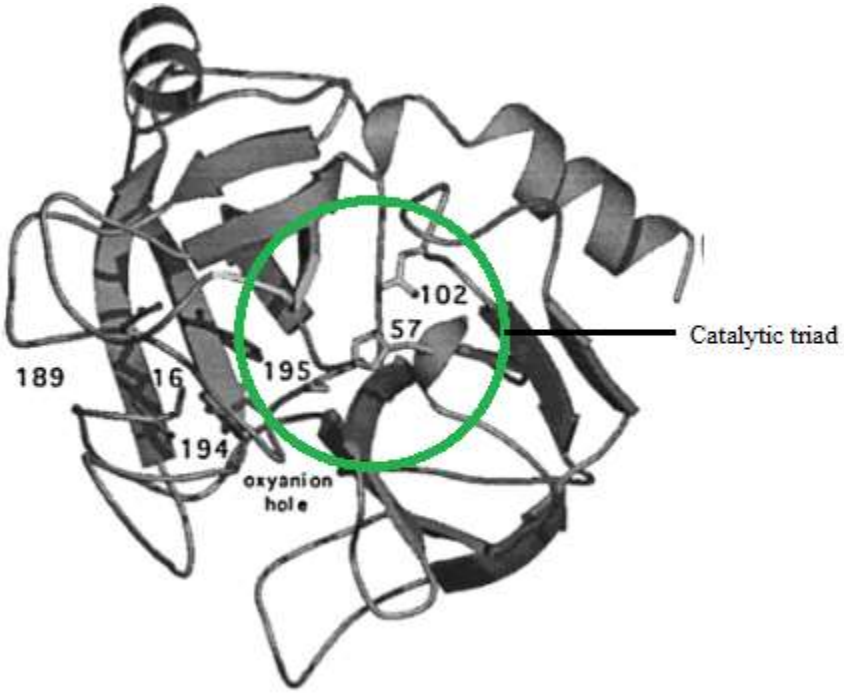


Figure 3.4. Structure of serine protease (Chymotrypsin) (Hedstrom, 2002).

(Tang and Wong, 1987; Guruprasad et al, 1995). These types of proteases possess two aspartic side chains which are essential for their catalytic mechanism. Aspartic proteases are found in rice seedlings and their gene structure and expression helps to detect the protein features and phylogenetic relation. It also helps in the rice growth and development. Aspartic proteases have also been purified from the seeds of plants such as wheat, buckwheat, barley, sunflower (Chen et al., 2009).

Aspartic proteases play important role in the therapeutic approach for treating diseases such as HIV, malaria, hypertension and Alzheimer's disease (Friedman and Caflisch, 2009). Martins et al. (2003) cloned and sequenced aspartic proteases from *plasmodium chabaudi* (a rodent parasite). *P. chabaudi* was selected as it was experimentally significant for therapy drug design. The study helped in using aspartic proteases as drug targets for chemotherapy of malaria. Aspartic proteases have been isolated from Basidiomycete *Cliticybe nebularis* (gilled fungi). The proteases found in *c. nebularis* are potentially effective for various biological applications and drug designing (Sabotic et al., 2009).

3.4.5. Metalloproteases

Metalloproteases are involved with matrix and non-matrix proteins in the extracellular environment of organisms and hence called matrix metalloproteases (MMPs) (Nagase et al., 2006). MMPs include nine members in this class (Hansen et al., 1993). The connective tissues are also known as extracellular matrix. These proteases play an important degradation role in extracellular matrix (ECM) remodeling. Miyamoto et al. (2001) isolated two alkaline metalloproteases in *Alteromonas sp.* (marine bacterium) and in various gram-negative bacteria (*Aeromonas, Helicobacter, Schewanella, Vibrio and Xanthomonas*) showing the presence of metalloproteases.

MMPs are involved in various neurological processes like angiogenesis (new blood vessel growth), myelinogenesis (development of myelin sheaths), cell protection, axonal growth, repair processes, and termination of inflammation (Yong et al., 2001). Inflammatory diseases like rheumatoid arthritis (destruction of rheumatoid joints) and

many others are caused by uncontrolled activity of MMPs (Hansen et al., 1993). Giron et al. (2012) isolated two non-hemorrhagic metalloproteases from *Bothrops colombiensis* (venom). These proteases were characterized as thrombolytic agents, which dissolves fibrin clots. Metalloproteases were isolated and characterized from chicken epiphyseal cartilage matrix vesicles and used to degrade non-collagenous protein moieties, which inhibit precipitation of minerals and thereby facilitating mineralization (Katsura and Yamada, 1986).

3.4.6. Cysteine Proteases

3.4.6.1. Occurrence: Cysteine proteases (CPs) are widely distributed among invertebrates (viruses, plants, protozoa, fungi and insects) and vertebrates (mammals) (Otto, 1997; Oliveira et al., 2003). CP's are also known as thiol proteases (Otto, 1996). In vertebrates, CP's control lysosomal protein degradation and aid some metabolic disorders. They act as digestive enzymes in the case of invertebrates (Oliveira et al., 2003). The papain family is considered major group in CPs. Capases and picornviridae families are the other groups of CPs. These proteases have a close similarity in their structure (Leung et al., 2000). Occurrence and examples of CPs are shown in Table 3.6.

A papain like cysteine protease was isolated by Enekel and Wolf (1993) from yeast and was found responsible for resistance against bleomycin hydrolase. The isolated protease is thiol-dependent enzyme. Mitchel et al. (1970) isolated papain from latex of *caica papaya* (melon tree). Cathepsin, a cysteine protease was isolated and characterized from American lobster gastric fluid (Rojo et al., 2010). The isolated cysteine protease showed the absence of polyproline loop in their structure, which is related with their role in digestion.

3.4.6.2. Proteolytic activity: Like serine proteases, CPs forms a catalytic triad to perform its proteolytic activity. Thiolate-imidazolium ion pair was formed by the catalytic triad between cysteine and histidine. The proteolysis takes place with four steps; (a) stabilization of hydrogen bond, (b) acylation and release of fragment in C-terminus, (c) regeneration of free enzyme and (d) Release of substrate fragment in N- terminus (Leung et al., 2000; Otto, 1996)..

Table 3.6. Occurrence and examples of Cysteine Proteases (CPs) (Otto and Schirmeister, 1997)

Occurrence	Examples
Viral cysteine proteases (rhinoviruses, poliomyelitis, and hepatitis A viruses)	Trypsin, chymotrypsin
Bacteria *(Staphylococcus aureus, Clostridium histolyticum)*	Clostripain, Arg-gingipain
Fungi *(Aspergillus flavus, Streptococcus thermophilus, Lactococcus lactis)*	Cathepsin B
Protozoa and worms *(Trypanosoma cruzi, Trichomonas vaginalis)*	Cruzipain
Plants *(Carica papaya*, *Carica papaya*, *Carica)*	Papain, ficin and bromalein
Mammals and humans	Cathepsin I, II and III,

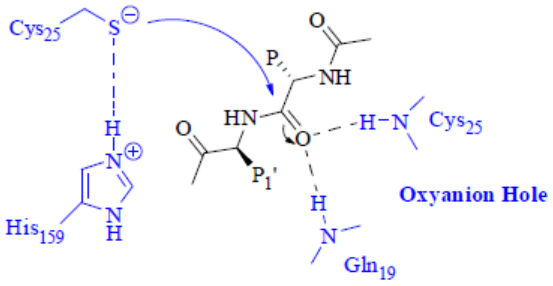
The four step proteolysis is shown in Figure 3.5. However, altered expression leads to muscular dystrophy, muscle denervation and tenotomy, hypertension and abnormalities in blood platelet (Johnson, 1990).

The role of Cysteine proteases in extracellular proteolysis was studied by Bromme and Wilson (2011). It was proven that the proteolytic activity of cysteine proteases were optimum at lysosomal acidic and reducing environment, at which the degradation of extracellular matrix (connective tissues) can be degraded. Improper regulation of cysteine proteases can release toxic peptides, which cause protein destruction and supportive connective tissues (Fritz et al., 1986). Johnson (1990) stated that calpains (calcium activated cysteine protease) showed limited proteolytic activity.

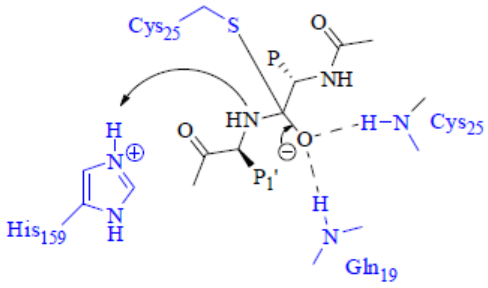
3.4.6.3. Functions: CPs have many physiological functions and improper regulation leads to many diseases. The physiological functions include: cell motility, proteolytic modification for signal transduction pathways and regulation of gene expression (Aoyagi et al., 1969).

The deregulation of CPs was considered the reason for all the above mentioned diseases and disorders. There are several factors that lead to the improper functioning of CPs they are: (a) pH – CPs are weak and unstable at neutral pH levels, (b) redox environment – the active site is readily oxidized and hence CPs are active in reducing environment, (c) inactive precursor synthesis – random activation leads to accidental secretion, (d) targeting endosomes and lysosomes – major binding to target lysosomes causes secretary pathways and (e) cysteine protease inhibitors (CPIs) – presence of CPIs inhibits the CPs. Controlled inhibition will help in proper regulation of CPs function (Chapman et al., 1997).

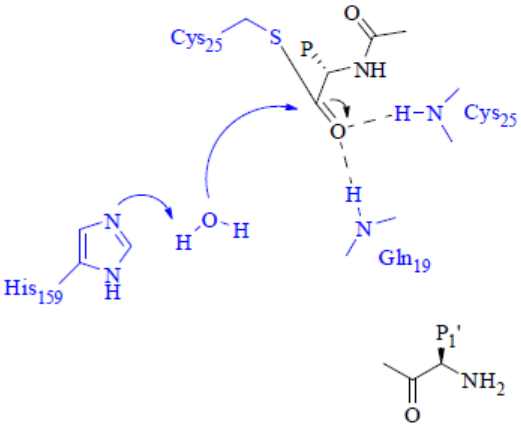
Omnipresence of CPs throughout the tissues helps in wound healing, bone remodeling and apoptosis (Nakamura et al., 2003, Dufour et al., 1995). The secretion of CP by bronchial epithelial cells leads to inflammatory airways disease. CP activates MMPs to break cartilage extracellular matter and thereby causing bone and joint disorders. Rapid activation of trypsin by CPs causes Acute pancreatitis (damage of pancreas by pancreatic enzymes) (Abbenante et al., 1996).



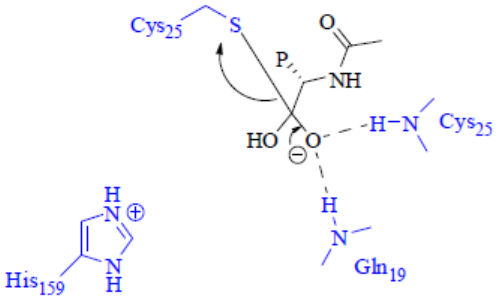
(a) Stabilization of hydrogen bond



(b) Acylation



(c) Regeneration



(d) Release of substrate

Figure 3.5. Four step proteolysis by CPs (Leung et al., 2000).

Hyper-secretion of CPs in tumors leads to tumor metastasis (Sasaki et al., 1990; Chan and Golec, 1996) and improper activity of CPs can promote Alzheimer's disease (neuropsychological illness) (Tyndall et al., 2005). CPs cleaves crystallins in the eye leading to the aggregation of fragments to form cataracts (Hunter and Ludwig, 1962). Down regulation of CPs also causes gastric cancer (Bebetti et al., 2005). Pandey and Dixit (2011) reported that CPs plays a critical role in parasitic life cycle by controlling degradation of erythrocyte proteins (hemoglobin) which helps in antimalarial chemotherapy. The review by Chen et al. (2012) stated the importance of CPs in heart disease. Proper regulation of CPs controls the extracellular matrix (ECM) of the heart and helps in maintained biological processes related with heart. Dysregulation may cause myocardium-coronary-valve disease (CCVD). Wolters and Chapman (2000) reported on the importance of CPs in lung diseases. CPs regulates immune responses.

3.5. Protease Inhibitors (PIs)

3.5.1. Importance

As discussed earlier, the regulative functioning of proteases is much needed for various functions. The deregulation of proteases results in various adverse effects. The proper functioning of proteases is achieved by the inhibitors binding to specific proteases (Eijik, 2003). Groups of plasma proteins with ability to inhibit the enzymatic actions of proteases are termed as protease inhibitors (PIs) or enzyme inhibitors. PIs form the third largest functional group of plasma proteins in the body. Some of the inhibitions occur naturally and some are induced artificially for the regulation of proteases (Hibbetts et al., 1999). The PIs are widely distributed like proteases and play a major role in many biological processes (blood coagulation, apostatis, and cell cycle). PIs also help in treating human pathologies (inflammation, hemorrhage and cancer). PIs are also used for studying protein interactions and drug designing (Lingaraju and Gowda, 2008).

3.5.2. Types of PIs

Based on the mechanism of inhibition, the PIs are classified into two general groups: (a) non-specific inhibitors and (b) specific inhibitors.

3.5.2.1. Non-specific inhibitors: Non-specific inhibitors can inhibit proteases irrespective of the class to which they belong. The human Alpha macroglobulins are the only members of this group. Alpha macroglobulins are large proteins with a low specificity which facilitates their broad spectrum of activity with all 4 major classes of proteases (serine, aspartic, metallo and cysteine proteases). Alpha macroglobulins perform their inhibition by a trap mechanism of action in which the protease binds to its generic bait region resulting in limited proteolytic activity (Hibbetts et al., 1999). The trap mechanism action of alpha macroglobulin is shown in Figure 3.6.

3.5.2.2. Specific Inhibitors: Inhibitors capable of inhibiting only one of the 4 major classes of proteases are termed as specific inhibitors. Unlike the non-specific inhibitors, these types of inhibitors have a low molecular weight and a high specificity. These inhibitors are named after the class of protease they are inhibiting, serine protease inhibitors, aspartic protease inhibitors, metallo protease inhibitors and cysteine protease inhibitors (Hibbetts et al., 1999). Based on the mechanism of action, the specific protease inhibitors are classified as either irreversible or reversible inhibitors. The irreversible inhibitors do not have similar structures and bind covalently or non-covalently to the proteases. Irreversible inhibitors have limited applications. Reversible inhibitors block the enzymes from forming a new product. Usually they bind non-covalently and form a reversible equilibrium with the enzyme (Sharma, 2012). The reversible inhibitors are further divided into 3 types (a) competitive inhibitors, (b) uncompetitive inhibitors, and (c) non-completive (or mixed) inhibitors.

A general enzyme substrate reaction in the absence of inhibitor is shown below, where the enzyme (E) binds with a substrate (S) to form the enzyme substrate complex (ES) (Gan, 2005).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1)$$

The equilibrium constant (K_I) for dissociation of enzyme inhibitor complexes is given as, (Sharma, 2012)

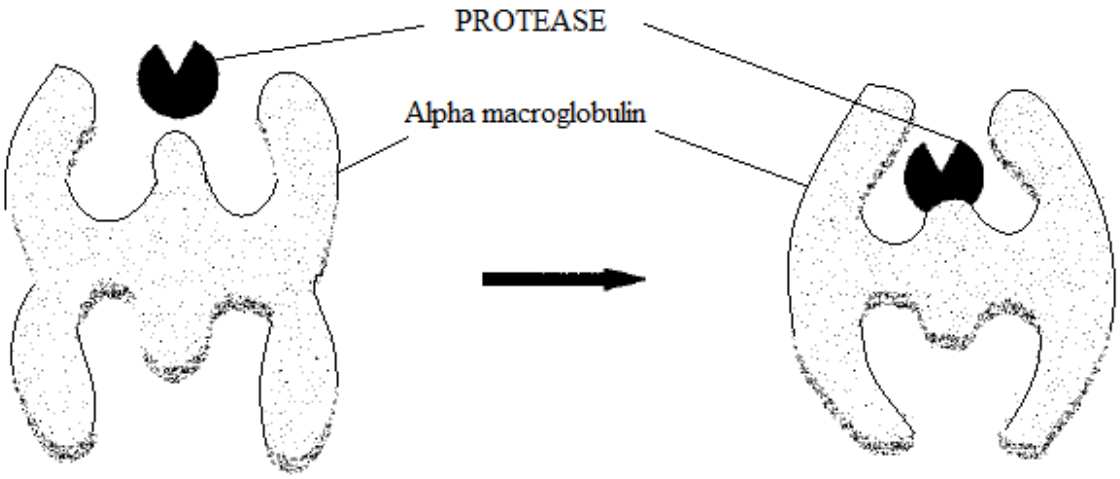


Figure 3.6. Trap-mechanism of action by alpha macroglobulin (Hibbetts et al., 1999).

$$K_I = \frac{[E]\,[I]}{[EI]} \qquad (2)$$

Competitive inhibitors have structure similarity and they reversibly bind to the enzyme's active site in a mutually exclusive manner with the substrate, thereby reduces the reaction rate (Sharma, 2012). Their reaction is as follows:

$$E + I \overset{K_I}{\rightleftharpoons} EI + S \longrightarrow \text{No product} \qquad (3)$$

Uncompetitive inhibitors do not have structural similarity and they bind either to the free enzyme (E) or the enzyme substrate complex (ES) away from the active site. These types of inhibitions cause structural distortion and stop the catalysis. Uncompetitive inhibition plays key roles in multi substrate reaction where the free enzyme reacts with two substrates (S_1, S_2). Multi substrate uncompetitive inhibition reactions are as follows (Sharma, 2012):

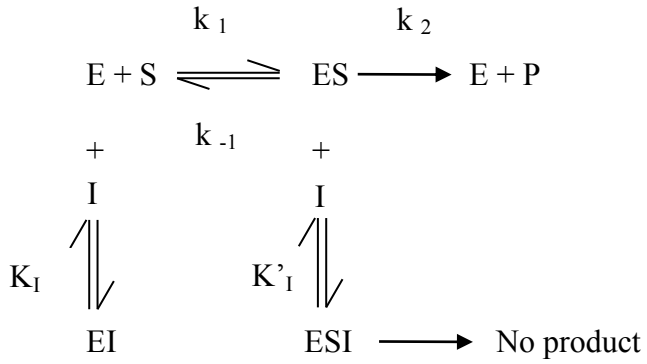
(a) Reaction with no inhibitor

$$E + S_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 + S_2 \overset{k_2}{\rightleftharpoons} ES_1S_2 \longrightarrow E + P_s \qquad (4)$$

(b) Reaction with inhibitor

$$E + S_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\,S_1 + I \overset{K'_I}{\rightleftharpoons} E\,S_1\,I \longrightarrow \text{no product} \qquad (5)$$

Non-competitive inhibitors (or mixed inhibitors) also do not have any structural similarity and they do not bind in a mutually exclusive manner with the substrate. They do not have any influence on the substrate and bind away from the active site. This type of inhibitor helps in altering the action of enzymes and thereby reducing catalytic activity. Their reaction is as follows (Gan, 2005; Sharma, 2012):



(6)

3.6. Cystatins – cysteine protease inhibitors (CPIs)

Cystatins are the inhibitors of papain and are related to CPs (Eijik, 2003). Cystatins have structural resemblance which is specific to the cysteine protease class (Oliveira et al., 2003).

3.6.1. Sources of Cystatins

Sources for cystatins include plants, animals and microorganisms. The physiological functions of cystatins are more explored and characterized in plants than in microorganisms and animals (Laskowski and Kato, 1980).

3.6.1.1. Plant Sources: Cystatins from plant sources are small proteins with high concentrations of total protein content (10%). Cystatins are mainly located in the storage tissues of plants (tubers and seeds). They are also found in the leaves and aerial parts of the plants by the actions of insects and micro pathogens (Ryan, 1990). Cystatins provide protection for plants by their defense mechanism. Plant sources are more explored for cystatins than the animals due to their medicinal value (Dunaevsky et al., 1998). Rice cystatins are the most studied because of their high protein content as they are highly heat stable (Abe and Arai, 1985). They are also found in chestnut fruit and pearl millet and they exhibit high antifungal activity (Bijina, 2006). Cysteine proteases found in the insect larvae are inhibited by cystatins (both synthetic and natural).

. Some important plant sources of cystatins, their target enzymes and functions are shown in Table. 3.7

3.6.1.2. Animal Sources: Cystatins form the largest group of natural PIs found in animal sources (Bode et al., 1988). They act by blocking the unwanted proteolytic processes of proteases in animals (Garcia-Oimedeo et al., 1987). Cystatins extracted from plant sources have good specificity towards the proteases in animal sources and cystatins from plant sources are also employed in animals (Roston, 1996). Cystatins provide protection against the action of bacteria, viruses and insects (Bijina, 2006). Various animal sources for cystatins and their properties are tabulated in Table 3.8.

3.6.2. Families of Cystatins

The diversity of cystatins has led to the evolution of the cystatin super family. Cystatins comprise of four families: (a) Family 1 or stefin family, (b) Family 2 or cystatin family, (c) Family 3 or kininogen family and (d) Family 4 or phytocystatins. Among the four families, family 4 cystatins or phytocystatins belongs to plants and the first three families (stefin family, cystatin family and kininogen family) belong to mammals (Otto, 1996; Habib and Khalid, 2007; Olieivera et al., 2003. The amino acid sequences, position of disulfide bonds are shown in Figure 3.7.

3.6.2.1. Family 1 Cystatins (Stefins): Cystatins belonging to this family have a molecular mass of 11,000 Da. They are found in the cytosol and do not possess neither disulfide bonds nor carbohydrate groups. Molecules of these cystatins contain 100 amino acid residues (Machleidt et al., 1983). Examples include cystatin A (human epithelial cells and neutrophilic granulocytes), cystatin B (all human cells and tissues), cystatin α and β in rats and recently stefin C from bovine thymus (Otto, 1996).

Brzin et al. (1983) isolated stefin from human polymorphonuclear granulocyte cytosol and found the isolated stefin to be capable of inhibiting papain, cathepsin B and H a 1:1 molar ratio and showed stability at high temperature and alkaline pH. Ebert et al. (1997) investigated the human lung tissue specimen and reported the presence

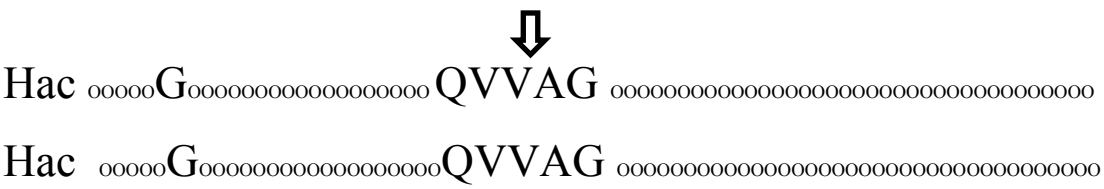
Table 3.7. Source, target enzyme and function of plant cystatins (Bijina, 2006).

Source	Target enzyme	Function
Oryza sativa	Cysteine proteinase	Heat stable
Pennisetum glaucum L.	Cysteine proteinase	High antifungal activity
Chestnut fruit	Cysteine protease	Antifungal activity
Penntsetum glaucum L	Cysteine protease	Antifungal activity

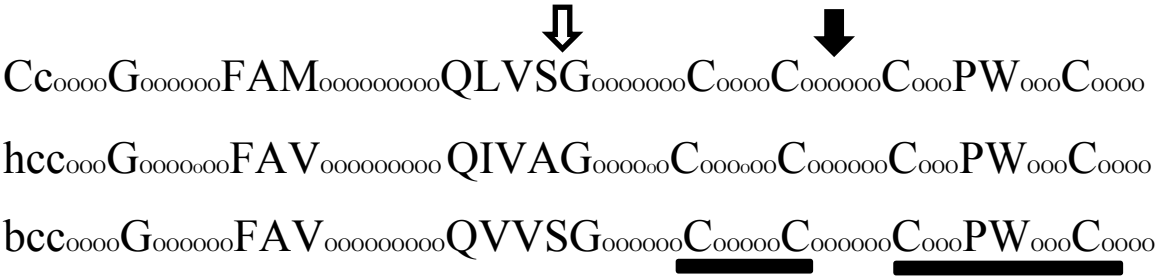
Table 3.8. Animal sources of cystatins and their action.

Source	Inhibitor	Action	References
Lung of *Capra hircus* (goat)	Thiol protease inhibitor (TPI)	Provides high immune response	(Khan and Bano, 2008)
Pancreas of *Capra hircus* (goat)	Thiol protease inhibitor (TPI)	Fights against pancreatitis (inflammation in pancreas)	(Priyadarshini and Bano, 2009)
Bovine skeletal muscle	Muscle cysteine proteinase inhibitor (MCPI)	Exhibits physiological properties in living cells	(Bige et al., 1985; Berri et al., 1996)
Bovine brain	Cysteine proteinase inhibitor (CPI)	Provide regulatory mechanisms for bioactive proteins and peptides	(Aghajanyan et al., 1988)
Human liver	Cystatin-like cysteine proteinase inhibitor (CPI)	Controls intracellular proteolysis	(Green et al., 1983)
Skin of Atlantic salmon	Tromsin I, Tromsin II	Protects cells from unfavorable intracellular proteolysis	(Synnes, 1998)
Larval hemolymph of tobacco hornworm	Cysteine proteinase inhibitor (CPI)	Protects against infection caused by bacteria and viruses	(Miyaji et al., 2007)

Family- 1 cystatin = stefin family

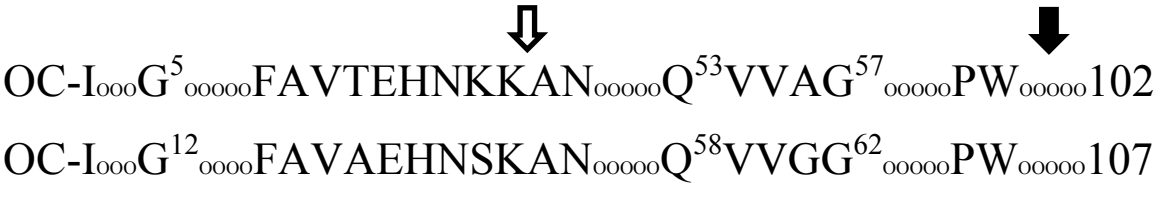


Family- 2 cystatin = cystatin family



Family- 3 cystatin = Kininogen family

Family- 4 cystatin = Phytocystatin



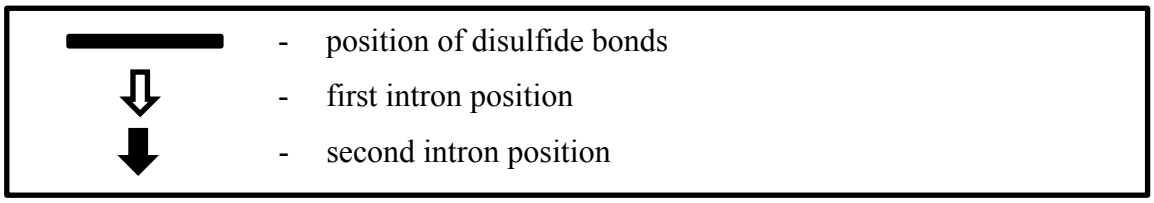


Figure 3.7. Alignment of amino acid series of the four families of cystatins (Oliveira et al., 2003).

of stefin A and stefin B. It was concluded that the high concentrations of both stefins (A and B) showed inhibitory activity against primary lung tumor.

3.6.2.2. Family 2 Cystatins (only Cystatin Family): Cystatins of this family are smaller when compared with family 1 cystatins. The molecular mass ranges from 13,000 – 24,000 Da with 115 amino acid residues (Abranhanson et al., 1987). Family 2 cystatins possess two disulfide loops at C-terminal, but do not have carbohydrate residues (except cystatin C from rat). They have conserved amino acid sequences which help in bindingwith target proteases (Machleidt et al., 1983). These inhibit plant cysteine proteases and cathepsins (Otto, 1996).

According to Whipple et al. (1990), calpastatin (a family 2 cystatin) can be used as a marker to predict meat tenderness. Goll et al. (2003) reported the importance of cystatins in gene regulation. Cystatins inhibit calpain activity and thereby maintain regulation in gene expression. Inomata et al. (1993) isolated and compared the properties of cystatins from rat erythrocytes and suggested the presence of cystatins in humans for maintaining gene regulation.

3.6.2.3. Family 3 Cystatins (Kininogen Family): The kininogen families are the largest of the mammalian group of cystatins and they are sub- divided into three types: (a) kininogens with high molecular weights (HMW) ranging from 60,000 – 120,000 Da and 355 amino acid residues, (b) kininogens with low molecular weight (LMW) ranging from 50,000 – 80, 000 Da and (c) kininogens with molecular weight 68,000 Da. The kininogen family has been identified in rats and not in humans. Cystatins of this family are capable of blood coagulation (Otto, 1996; Oliveira et al., 2003).

Thompson et al. (1978) isolated high molecular weight (HMW) kininogens from pooled human plasma and stated that the isolated kininogen showed an effective coagulation activity due to the presence of a heavy and light chain linked by a disulfide bond in their structure. A HMW kininogen was also isolated from horse plasma by Sugo et al. (1981) who reported similarities with kininogen extracted from bovine. However, the horse plasma kininogen were immunologically different. Hermann et al. (1996)

studied the expression and cellular localization of kininogens in human kidney and stated that it helps in regulation of renal blood flow and electrolyte excretion.

3.6.2.4. Family 4 Cystatins (Phytocystatins): Phytocystatins include almost all the plant cysteine proteinase inhibitors (Oliveira et al., 2003). Barret et al. (1986) reported the first phytocystatin (oryzacystatin) from rice seeds. They had homologous structure and function as chicken egg white cystatin. The plant cysteine proteinase inhibitor classifications include: (a) phytocystatins (single domain with majority of phytocystatins) and (b) multicystatins (multiple domains from potato tubers). Phytocystatins include both monocot and dicot species (Abe et al., 1987; Walsh and Strickland, 1993).

Ryan et al. (1998) purified phytocystatins from apple fruit (*Malus domestica*) and characterized it as an endogenous inhibitor which acts against pest or microbial attack. An extracellular insoluble phytocystatin was isolated by Ojima et al. (1997) from cell cultures and carrot seeds. They suggested the use of isolated phytocystatins for somatic embryos and also control proteases for regulated seed germination. Kouzuma et al. (1996) performed the purification of phytocystatins from sunflower (*Helianthus annuus*) seeds and stated that they were identical to animal cystatins.

3.6.3. Interaction with Target Proteases

Each class of PIs reacts with their target proteases by different interaction mechanisms. These mechanisms usually differ with each class-specific PI. The mechanism of cystatins differs from serine, aspartic and metallo PIs. Cystatins are exosite binding inhibitors, which bind adjacent to the active site blocking the substrate. They do not interact with the free enzyme directly (Bode and Huber, 2000; Bijina, 2006). Cystatins utilize a steric blockage mechanism for substrates. The inhibitory mechanisms of cystatins are of several types: (a) blocking of active site by backward binding, and distortion of active site, (b) blockage of active site, (c) blockage of active site with partial substrate binding and (d) blockage by backward binding and blockage of active center by substrate-like binding (Rzychon et al., 2004) Diagrammatic representation of these types are shown in Figure.3.8.

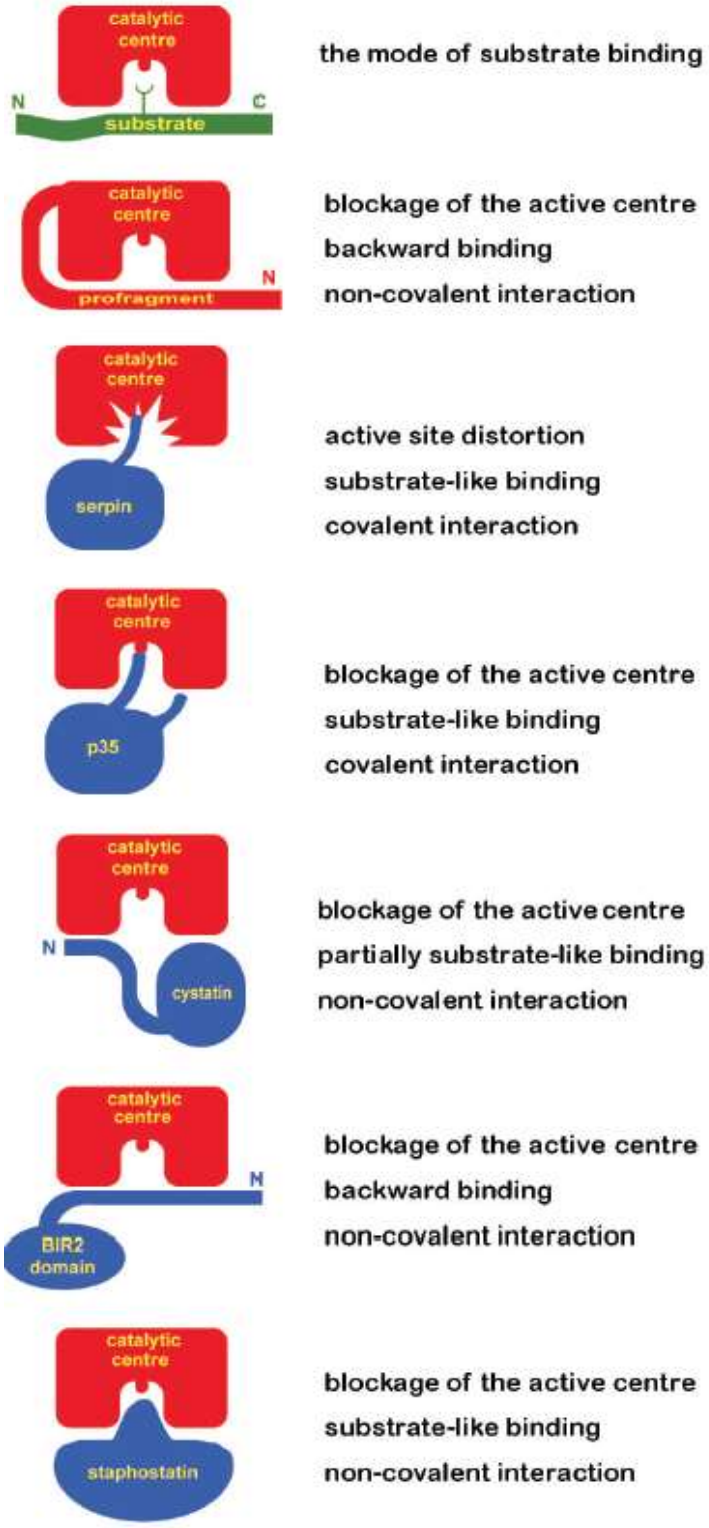


Figure 3.8. Inhibitory mechanisms of cystatins (Rzychon et al., 2004)

The interaction of cystatin and papain-like cysteine proteases can be studied for the complete understanding of the cystatin mechanism. Cystatin subsites itself adjacent to the active site during its interaction with papain-like cysteine inhibitors. The N-terminal segment bridges over the active site and P2 and P1 residues of cystatin are placed in subsites (S1, S2) (Bode and Huber, 2000). Schematic representation of cystatin interaction with papain-like cysteine protease is shown in Figure. 3.9.

3.6.4. Applications

Cystatins are capable of regulating pathological processes and hence they have wide applications against various diseases related with cardio, kidney, liver and neurological disorders (Bobek and Levine, 1992). Due to their varied applications for diseases they are studied for drug development. The purified forms of cystatins are used in drug designing for treating cancer and other inflammatory diseases (Otto, 1996).

3.6.4.1. Role in Cardiovascular Diseases (CVD): Cystatins play a key role in diagnosing heart failures in the population of elderly people. The mechanism by which cystatins serves to interpret heart failures is still unclear. However, the increase in cystatin concentration predicts the secondary cardiovascular events (Brgulijan and Cimerman, 2007). Cardiovascular disease (CVD) has become an important cause of death. Commonly known reasons for CVD are diabetes, hypertension and smoking. However, some CVDs are caused by renal disorders. Cystatin C (low molecular weight cystatin) is employed in the diagnosis of CVDs and they have proved to be highly effective. People with CVDs, which are not caused by common factors like smoking and hypertension, can benefit from the application of cystatin C as biomarkers. Studies by Cepeda et. (2010) proved the association of cystatin C with CVDs.

3.6.4.2. Role in Diseases Related with Kidney: The detection and control of chronic kidney diseases has gained much attention, due to the increase of diseases especially in the elderly population. The Glomerular filtration rate (GFR) technique is the most popular method used in the detection of chronic kidney diseases. GFR uses different

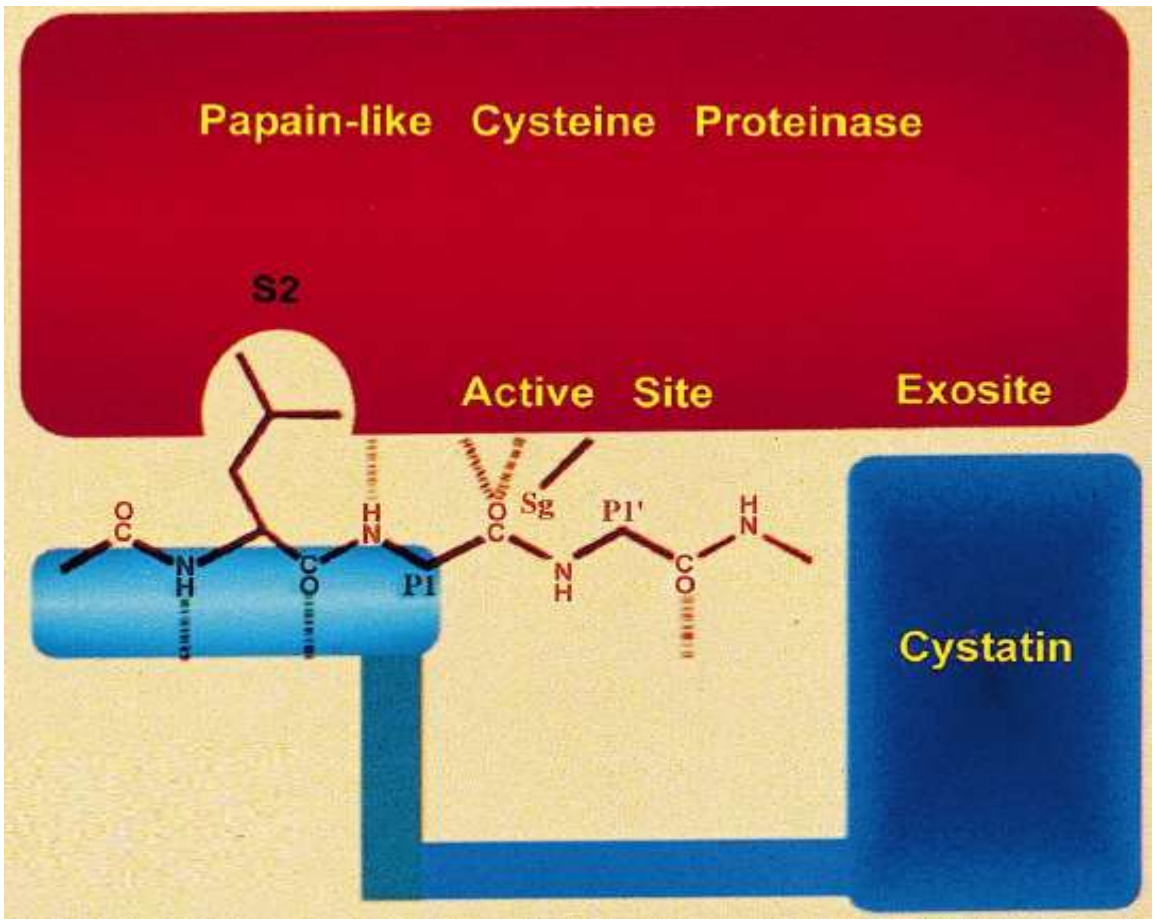


Figure 3.9. Cystatin interaction with papain-like cysteine proteinase (Bode and Huber, 2000).

markers such as creatinine. Cystatin C serves as an efficient marker for GFR (Brgulijan and Cimerman, 2007).The initial discovery for the use of cystatin C in GFR was slow and the results were close in comparison with creatinine. Later, the advancements in science and comparative studies on cystatin C and creatinine improved the process. This led to increased efficient usage of cystatin C in GFR (Grubb, 2010).

Jernberg et al. (2004) studied the effect of cystatin C on patients with suspected or confirmed acute coronary syndrome and provided substantial proof that they improve early risk stratification of the patients. Larsson et al. (2005) investigated the effect of increased cystatin C in elderly population and it was found to be a stronger predictor of the risk of death. Peralta et al. (2011) reported that cystatin C is a stronger predictor of chronic kidney disease (CKD) and it can be used for identifying CKD for persons with high risk complications.

3.6.4.3. Role against Liver Diseases: Rheumatoid arthritis (autoimmune disease) principally affects the flexible joints, but also causes liver inflammation. It can be monitored by measuring cystatin C levels. Cystatin C has been proved as a better marker than creatinine for liver disorders (Brgulijan and Cimerman, 2007). Liver disorders are of great risk as they may cause serious inflammations, necrosis, liver cirrhosis and hepatoma. Liver fibrosis was found to be a cause for liver diseases by degradation of extracellular matrix. Cysteine proteases are responsible for liver fibrosis. Diseases like hepatoma need long term monitoring and hence the marker must be highly stable. Cystatin has been proven to be an efficient tool for treating against liver diseases by inhibiting exogenous cysteine proteases, which are responsible for liver disorders (Chu et al., 2004).

Takeuchi et al. (2001) explored the clinical possibility of using cystatin C for chronic liver diseases. Their results showed that increases in cystatin C concentration increased the progression of chronic liver disease. Hence, it can be used as an effective marker for liver fibrosis. Ladero et al. (2012) analyzed serum cystatin C for use as a non-invasive marker for chronic hepatitis C. Their results stated that it may help in detecting liver fibrogenic activity and cannot be used as non-invasive marker of liver fibrosis. Samyn et

al. (2005) reported that cystatin C is a reliable marker for the assessment of liver diseases and renal dysfunction in children after liver transplant (LT).

3.6.4.4. Role in Neurological Disorders: Amyotrophic lateral sclerosis (ALS), a fatal neurologic disorder is caused by degeneration of motor neuron. The number of people affected by this disease throughout the world is increasing. The abundance of cystatin C in the cerebral spinal fluid makes them the most efficient biomarker for ALS disease. Cystatin C helps to differentiate ALS disease and ALS mimics. Studies show that cystatin C is involved in the pathogenesis of ALS (Wilson et al., 2010).

D'Adamio (2010) reported that cystatins have been implicated for neuronal degeneration and repairing the nervous system. Cathepsins involved in housekeeping functions are regulated by reversible cystatins (CysC and CysB). CysC are neuroprotective and prevents neurodegeneration at optimal concentrations. Neurological disorders like Alzheimer's disease have no preventive or curative drugs. CysC has been considered for designing drugs for such neurological disorders

Xu et al. (2004) stated that the damage caused to nigrostriatal dopaminergic (DA) pathways causes inflammation and increased synthesis of neural growth factors. These eventually cause damage for regeneration of the central nervous system (CNS). Disorder caused due to the destruction of DA pathway is referred as Parkinson's disease. The changes made in the expression of cystatin helps in limiting neuropathy in Parkinson's disease.

3.6.4.5. Role in Cancer Treatment: Bell-McGuinn et al. (2007) reported that increased protease expression and activity leads to progression of tumor cells. Cancer research results shows that cystatins can be used for the treatment of cancer in combination with chemotherapy. The results displayed regression of tumor growth and increased overall survival. The inhibition of cathepsin is needed for reduction of tumor invasion. Cystatins in combination with maximum tolerated dose (MTD) or metronomic dose of cyclophosphamide helps in the reduction of tumor cell proliferation. Results have led to continuous development and evaluation of cystatins as therapeutics for cancer.

Sokol and Schiemann (2004) studied the effect of cystatin C on normal and cancer cells. They stated that cystatin C is an effective tumor suppressor and inhibits cell cycle progression leading to the prevention of cancer progression. Veena et al. (2008) reported that cystatin E/M suppresses cervical cancer and it was facilitated by the inhibition of cathepsin L (cysteine protease) by cystatin E/M. Sokol et al. (2004) studied the use of cystatin C to inhibit epithelial-mesenchymal transition (EMT) and suggested that it can be used in therapeutic response of human breast cancer.

3.6.4.6. Role in Mammalian Parasitic Disease Treatment: Therapeutically effective doses of cystatins have been used in the treatment of mammalian parasitic diseases (Black and Beaulieu, 2010). These parasitic diseases include toxoplasmosis, malaria, African trypanosomiasis, Chagas disease, leishmaniasis or schistosomiasis (Black et al., 2008). Chagas and leishmaniasis diseases are the most commonly treated with the application of cystatin.

Chagas disease is caused by the parasite *Trypanosoma cruzi* and is responsible for cardiomyopathy (heart muscle disease). As of 1990, 12 million people have been infected in 15 Latin American countries. People undergoing organ transplants and certain cancer treatments have a high risk of developing this disorder. Hence treatment of the disorder is very much necessary. Inhibition of cruzain (the target protease) is needed for treating the disorder, which can be achieved by cystatin treatment. Promising results have been shown by cystatins over the other drugs used with limitations and cystatin treatment is in the late stage of preclinical development (Doyle et al., 2007). Silva et al. (1995) isolated cystatin S from submandibular gland of infected rats and reported that it can be used as specific inhibition therapy for Chagas disease. Nathanson et al. (2002) studied the regulated expression and intracellular localization of cystatin F. Results showed that cystatin F can inactivate a family 1 target enzyme which is a virulence factor of Chagas disease.

Visceral leishmaniasis (kala-azar or black fever) is caused by the *Leishmania* genus and considered the largest lethal parasitic disease after malaria. Cystatin helps in complete cure of leishmaniasis, when associated with up regulation of Th 1 cytokine and

inducible nitric oxide synthase (iNOS). The drug is in later stage of preclinical trials (Kar et al., 2011). Das et al. (2001) reported on a successful therapy of lethal murine visceral leishmaniasis with cystatin and stated that cystatin possess stimulatory capacity for enhanced macrophage activation leading to the treatment of leishmaniasis. Kar et al. (2009) studied the curative effect of cystatin on visceral leishmaniasis and their results showed enhanced inhibition of parasite growth.

3.6.4.7. Role in Treatment of Hookworm Infection: Hookworm disease is a soil-transmitted disease and it is a major global health problem causing chronic disability with 10% of the sub-Saharan African population affected by this disease. Treatment and control of this disease includes processes such as de-worming with established drugs. Cystatin stand as a distinctive drug for curing the hookworm disease with striking efficacy. Cystatin helps in eliminating the hookworm through the bloodstream. The drug is in the initial stage of preclinical trials (Vermeire et al., 2012). Wongkham et al. (2005) performed cystatin enzyme-linked immunosorbent assay against cysteine proteases and reported that it can be used for treating hookworm infections. Loukas and Prociv (2001) reported that cystatins are not found in hook worms but can be used against hookworm infections.

3.7. Extraction of Cystatin

Bovine skeletal muscle, human liver, bovine brain, rat liver, goat lung and pancreas are used to isolate and characterize cystatins. Exaction of cystatins from various animal sources is achieved by two techniques: (a) acetone fractionisation, (b) ammonium sulphate fractionisation

3.7.1. Extraction of Cystatin by Acetone Fractionation

Martínez-Maquenda et al. (1980) used the acetone precipitation technique for protein recovery for the extraction of cystatin from animal sources. The Acetone fractionation method included: (a) homogenization of the sample, (b) centrifugation for 30 min at 2000g and 4° C, (c) adjusting the pH of supernatant to 11 with 3 mM NaOH, (d)

incubation at 4° C for 2h, (e) adjusting the pH to 6.5 with 2M- HCl, (e) addition of acetone, (f) centrifugation for 30 min at 2000g and 4° C, (g) centrifugation of the supernatant, (h) suspension of the pellet in appropriate buffer and (i) purification. The experimental design for acetone fractionisation is shown in Figure 3.10.

Green et al. (1983) extracted cystatins from human liver using acetone fractionation technique. Two CPIs (CPI-A and CPI- B) were extracted and characterized. The extraction process involved: (a) alkaline denaturation, (b) acetone fractionisation, (c) affinity chromatography and (d) chromatofocusing. Frozen liver tissue was thawed, cut into pieces (1 cm^3) and homogenized with buffer. The precipitate was removed by centrifugation and treated with alkali to a pH of 11.0. Acetone fractionisation was performed after alkali treatment. The purification procedure was carried out by affinity chromatography. The extracted CPIs resembled egg-white cystatins.

Warwas and Sawicki (1989) used a similar acetone fractionation technique for the isolation of cystatins from human placenta. The purification was carried out with gel-chromatography on a Sephadex G-75 column.

Aghajanyan et al. (1988) used a acetone fractionation method to isolate cystatins from bovine brain. Fresh bovine brain cortex was washed with 0.15 M NaCl and homogenized with a suitable buffer in a blender. It was then treated with alkali and fractionized with acetone. Purification was performed with Sephadex G-75 column.

3.7.2. Extraction of Cystatin by Ammonium sulphate precipitation

King (1972) used the ammonium sulphate precipitation method for protein recovery for the extraction of cystatin. The precipitation processes included: (a) homogenization, (b) centrifugation for 15 min at 5000 rpm in 4° C, (c) adjusting the pH to 11 with 3mM NaOH, (d) incubation for 30 min at 4° C. (e) adjusting the pH to 7.5 with acetic acid, (f) centrifugation for 15 min at 10000 rpm and 4° C, (g) dissolving the sample in ammonium sulphate solution, (h) centrifugation for 15 min at 10,000 g, (i) addition of ammonium sulphate solution to reach final saturation and (j) dialysis to remove

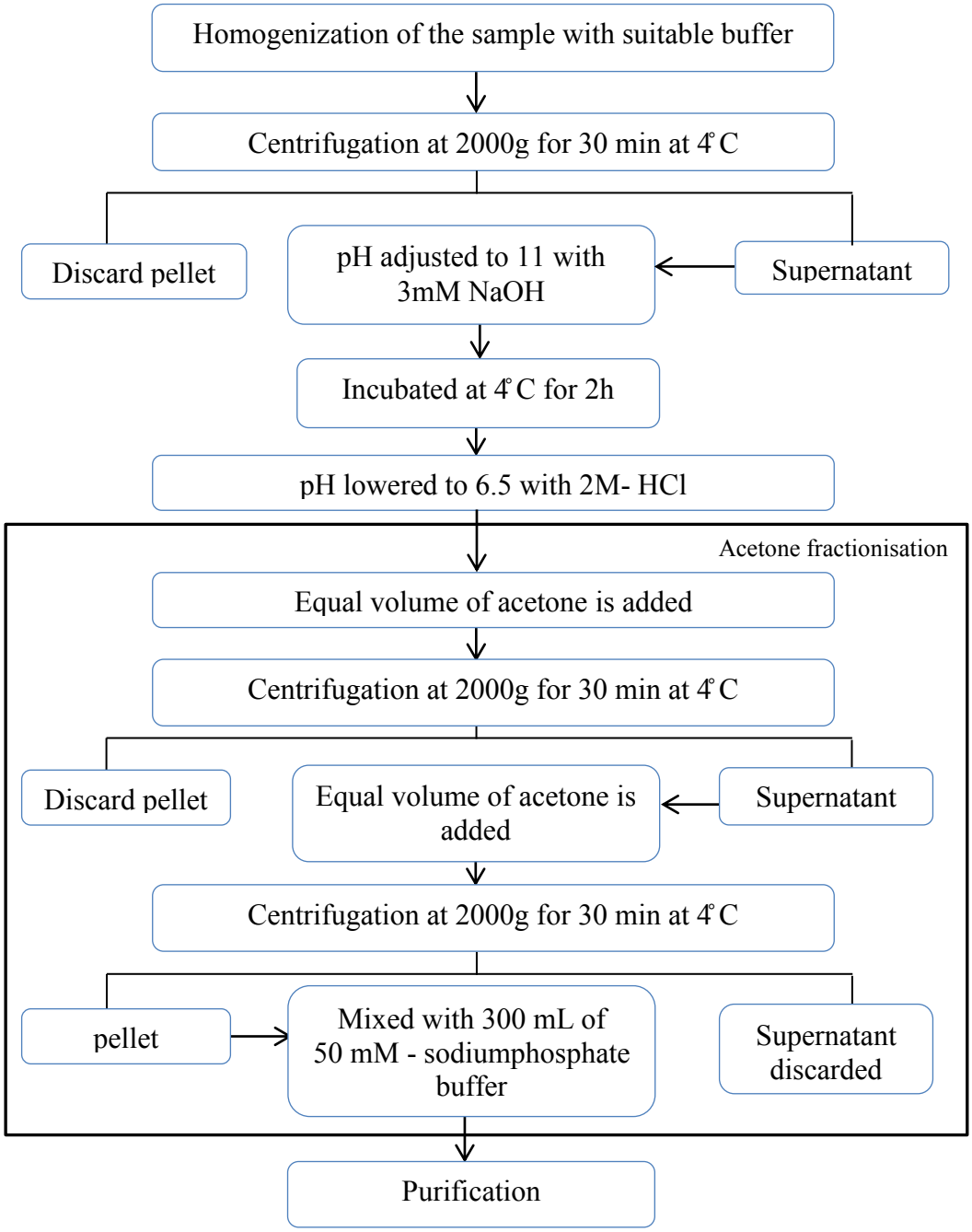


Figure 3.10. Cystatin recovery by acetone fractionation (Green et al., 1983).

additional salt content (Kunitz, 1947; Khan and Bano, 2008). The procedure is shown in Figure 3.11.

Khan and Bano (2008) used an ammonium sulphate precipitation procedure to extract cystatin from goat lung. The extraction buffer used consisted of 1% NaCl, 3 mM EDTA, and 2% n-butanol. 100g of the goat lung was cut into pieces and homogenized with 200 mL of the extraction buffer. The homogenized mixture was centrifuged at 5000 rpm for 15 min at 4° C. The pH was adjusted to 11 with 3mM NaOH and the pellet was discarded. The supernatant was incubated for 30 min at 4° C. After incubation, the pH was changed to 7.5 with acetic acid and then centrifuged at 10,000 rpm for 15 min at 4° C. The supernatant was made to 40% saturation by the addition of ammonium sulphate and the pellet was discarded. The sample was centrifuged again at 10,000 rpm for 30 min at 4° C. and then made up to 60% saturation by adding the required amount of ammonium sulphate. The sample was then centrifuged at 10,000 rpm for 15 min at 4° C and the supernatant was discarded. The pellet was dissolved with the suitable solvent. Dialysis was performed by treating the sample against 1% NaCl, 3 mM EDTA, and 2% n-butanol. The dialyzed sample was taken for purification. Ion-exchange chromatography was performed using a DEAE - cellulose column (2 × 7 cm) equilibrated with 50 mM sodium phosphate buffer at pH 8.0, and the proteins were eluted by a linear gradient of 0-0.5 M NaCl using 50 mM sodium phosphate buffer, pH 8.0. The fractions were collected and tested for protein concentration and inhibitory activity

Priyadarshini and Bano (2009) used an ammonium sulphate precipitation method to extract cystatin from goat pancreas. The extraction buffer (pH 7.5) used consisted of 50 mM sodium phosphate buffer (200 mL), containing 0.15 M sodium chloride (NaCl), 3 mM ethylene diammine tetra acetic acid and 2% n-butanol. 100g of goat pancreas was homogenized with the extraction buffer and the sample mixture was centrifuged at 5000 rpm for 15 min at 4° C. The pH of the supernatant was adjusted to 11 with 3 mM NaOH and the sample was incubated for 30 min at 4° C. The pH was changed to 7.5 by adding acetic acid and the sample was made to 40% saturation with the addition of ammonium sulphate. The supernatant was again centrifuged at 10,000 for 15 min at 4° C. The saturation of the supernatant was then increased to 60 % by adding ammonium sulphate.

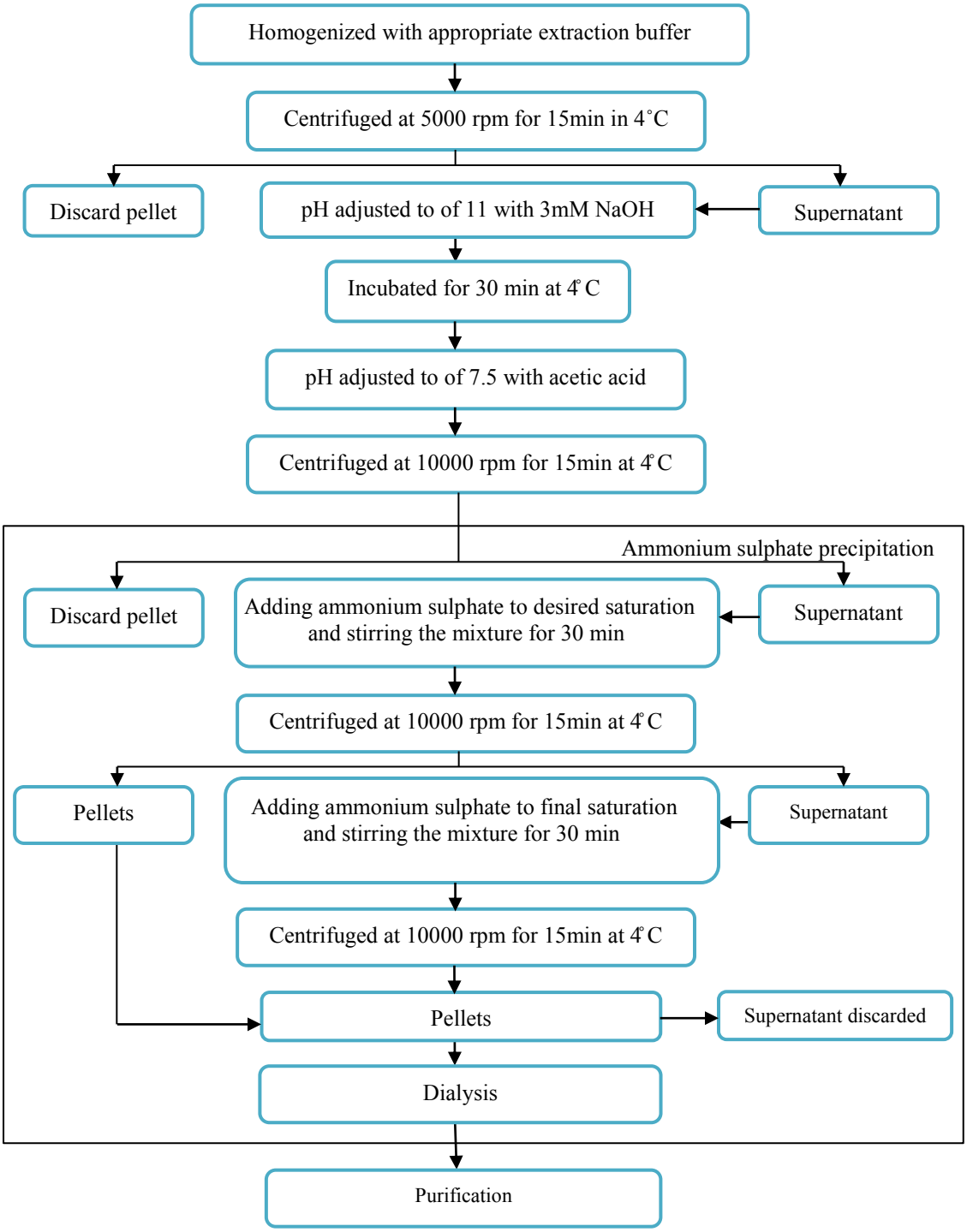


Figure 3.11. Cystatin recovery by ammonium sulphate precipitation (Khan and Bano, 2008).

The mixture was centrifuged at 10,000 for 15 min at 4° C and the pellet was discarded. The supernatant was centrifuged at 10,000 rpm for 15 min at 4° C. The supernatant was discarded and the pellet was dissolved with suitable buffer solution. The sample was then dialyzed with the extraction buffer. The sample was subjected to gel filtration chromatography on Sephacryl S-100 HR column (60 X 1.7 cm) equilibrated with 50mM sodium phosphate buffer (pH 7.5). The flow rate of the column was 15 mL h-1. A single protein peak with papain inhibitory activity was obtained corresponding to the goat pancreas thiol protease inhibitor. The fractions were tested for protein concentration and inhibitory activity.

The extraction of cystatin from bovine skeletal muscle using the ammonium sulphate precipitation technique was conducted by Berri et al. (1996). Ammonium sulphate saturation of 40-80% was performed. The purification procedures involved gel-chromatography on a Sephadex G100 column. And it was again purified by two successive anionic exchange chromatographies on Q-Sepharose and Mono Q columns. The characterization revealed the absence of disulfide bonds and the presence of monomer. Double immunodiffusion and Western blot techniques revealed the presence of cystatins in bovine heart, spleen, liver and lung. It was not found in bovine plasma (Berri et al., 1996).

3.8. Factors Affecting the Extraction Process

The extraction process is affected by several parameters including: incubation time, temperature, pH and ammonium sulphate saturation.

3.8.1. Incubation Time

The incubation time is adjusted for better yield and purity of the protein. Protein yield decreases with increase in incubation time in the denaturant. Prolonged incubation may cause failure in protein refolding (Dobson et al., 1998; Kathir at al., 2005) and may lead to protein denaturation, the native structure of protein (protein refolding) is altered due to the loss of bioactivity (Tanford, 1986; Vert et al., 2012).

Khan and Bano (2008) performed the extraction of cystatin (thiol proteinase inhibitor) from goat lung with 30 min incubation and recovered about 68% of cystatin after ammonium sulphate fractionisation. Priyadarshini and Bano (2009) used the same incubation time (30 min) for the purification of cystatins (thiol proteinase inhibitor) from goat pancreas and reported a yield of 58.8% after ammonium sulphate extraction.

Sadaf et al (2005) isolated, characterized and studied the kinetics of goat cystatins from the goat kidney. The homogenate was incubated for 10 min and a yield of 81% was obtained. Nuhayati et al. (2013) used the same incubation time (10 min) in the purification and characterization of cystatin (cathepsin inhibitor) from catfish and obtained a recovery yield of 17.45%

Aghajanyan et al (1988) purified cystatins from bovine brain. The purification step included alkaline treatment followed by acetone fractionisation and chromatography. The bovine brain homogenate was incubated for 2 h and the recovered yield was 31.8% after purification by chromatography. Trziszka et al. (2006) used one hour incubation time during the isolation of egg white cystatin and an inhibitory activity of 92.6% was observed.

3.8.2. Temperature

Temperature plays an important role in protein folding. Maintenance of temperature during incubation is important to recover biologically active proteins with high yield (Clark, 1998). At higher temperature (above 30º C) denaturation is endothermic and at low temperature (below 30º C) denaturation is exothermic (Tsai et al., 2002; Ascolese and Grazuano, 2008).

Brillad-Boudet et al. (1998) purified cystatin from Taiwan cobra (Naja naja atra) venom by incubating the homogenized sample at room temperature (21º C). Two forms of inhibitors were characterized after isolation. pI values of the extracted cystatins were 6.2 and 6.1.

Warwas and Sawicki (1988) isolated cystatin from human placenta by incubating the homogenized sample at 4º C. The purified cystatin was isolated with a yield of 13.50%.

Khan and Bano (2008) used the same temperature in the isolation of cystatin from goat lung and obtained a recovery yield of 58.8%.

Nuhayati et al (2013) incubated the homogenate at 80º C during the purification of cystatin from catfish and obtained a cystatin yield of 17.45% after precipitation.

3.8.3. pH

Protein unfolding is pH dependent, sufficient lowering of pH will facilitate proper protein folding with increased activity (Anderson et al., 1990). pH helps in the activation of enzyme and improper pH levels lead to decreased activity (Dickinson, 2002; Yang et al., 2007).

Green et al (1983) purified cystatins from human liver and lowered the pH to 6.5 after incubation. The molecular mass of the recovered cystatin was 12-KDa. Warwas and Sawicki (1988) isolated two low-molecular cystatins from human placenta. The isolation procedure included alkalization, acetone fractionisation and affinity chromatography. After incubation, the pH was set to 6.5 and a total activity (TA) of 15.20 Units was observed.

Hirado et al (1984) purified and characterized a low molecular weight cysteine proteinase inhibitor (CPI) from bovine colostrum. The pH for fractionisation was maintained at 7.4 and the recovery yield was 34.1%.

Berri et al (1996) studied the tissue distribution and characterization of cysteine proteinase inhibitor from bovine skeletal muscle. During the purification process a pH of 7.6 was maintained. The molecular mass of the recovered cystatin was ~ 30-KDa.

Turk et al (1992) isolated and characterized stefin B (CPI) from bovine thymus. The pH was adjusted and maintained at 7.5 during the isolation. The molecular mass of the recovered cystatin was 11-KDa.

Tsushima et al (1995) performed sequential chromatographies for the isolation, purification and the study of amino acid sequence of cystatin from bovine hoof. The pH was set to 8.0 and the recorded recovery yield was 64 %.

3.8.4. Ammonium Sulphate Saturation

Stigter et al. (1991) reported that salt concentrations are important in maintaining the stability of proteins. Improper concentration levels will weaken the proteins and result in lower yields. Salt concentrations are managed by changing the pH levels of the buffer to which the salt is mixed (Heller et al., 1997). Chen and Clark (2003) reported that protein stability is low at moderate concentration (< 200 mM) of salts and high at higher salt concentrations (> 200 mM).

Hirado et al (1984) used a saturation of 35-60% for the purification of low molecular weight CPI from bovine colostrum and recovered 6.3% of purified CPI.

Rawdkuen et al (2005) fractionized and characterized CPI from chicken plasma. They employed both PEG (poly ethylene glycol) fractionisation and ammonium sulphate fractionisation for the isolation process and recovered about 39.66% by ammonium sulphate fractionisation at 40% saturation which was higher than the yield of 37.70% recovered from PEG (poly ethylene glycol) fractionisation.

Berri et al (1996) used 40-80% saturation for extracting CPI from bovine skeletal muscle and the molecular mass of the recovered cystatin was ~ 30-KDa.

Khan and Bano (2008) fractionized the extract between 40-60% during the isolation of cystatin from goat lung and recovered a yield of 51.6%.

3.9. Purification

Column chromatography is the preferred technique for the purification of proteins (Still et al., 1978; Berg et al., 2002). The column chromatographic techniques used for cystatin purification include: (a) gel-filtration chromatography, (b) ion-exchange chromatography and (c) affinity chromatography.

3.9.1. Gel-filtration Chromatography

It is a widely used technique for protein separation and purification. The column is made of insoluble, highly hydrated polymer such as dextran or agarose or

polyacrylamide. The commercially available columns include: Sepharose, Sephadex and Bio-gel. These columns retain the smaller molecules and the larger molecules pass through the column (Hagel, 2001; Stanton, 2003). The fractionized sample is applied at the top of the column (Berg et al., 2002). The schematic representation of gel-filtration chromatography is shown in Figure 3.12.

Warwas and Sawicki (1988) purified cystatins from human placenta using gel-filtration chromatography. The column used for chromatography was Sephadex G-75 (2.2 X 41 cm) equilibrated with 0.1 M phosphate buffer (pH 6.0), 1.33 mM Na_2EDTA.

Hirado et al. (1984) purified cystatin from bovine colostrum using gel-filtration chromatography. The column used for chromatography was Sephadex G-50 (8 X 100cm) equilibrated with 20 mM sodium phosphate buffer (pH 7.0).

Takayiki et al. (2007) performed the purification of cystatin from larval hemolymph using gel-filtration chromatography. The column used was Sephadex G-50 (2.5 X 65 cm) equilibrated with 20 mM Tris-Hcl.

3.9.2. Ion-exchange Chromatography

The separation by ion-exchange chromatography is performed based on the net charge. Proteins with net positive charge (pH 7) bind to beads containing carboxylate groups and the negatively charged elutes (Skopes, 1987; Kenney, 1992).

Proteins with low density of net positive charge will be eluted first followed by the high charge density. Positively charged (diethylaminoethyl-cellulose (DEAE-cellulose) columns are used to separate negatively charged proteins and negatively charged (carboxymethyl-cellulose (CM-cellulose)) columns are used to separate positively charged proteins (Sharma and Ortwerth, 1994; Berg et al., 2002). Diagrammatic representation of ion-exchange chromatography is shown in Figure 3.13.

Nuhayati et al. (2013) purified cysteine inhibitor obtained from catfish (*Pangasius sp)* using ion-exchange chromatography. The column used in the purification was DEAE Sephadex A-75 (Sigma) equilibrated with 20 mM Tris base pH 7.5 containing 10 mM sodium azide (Merck) and 10 mM 2-mercaptoethanol (Sigma).

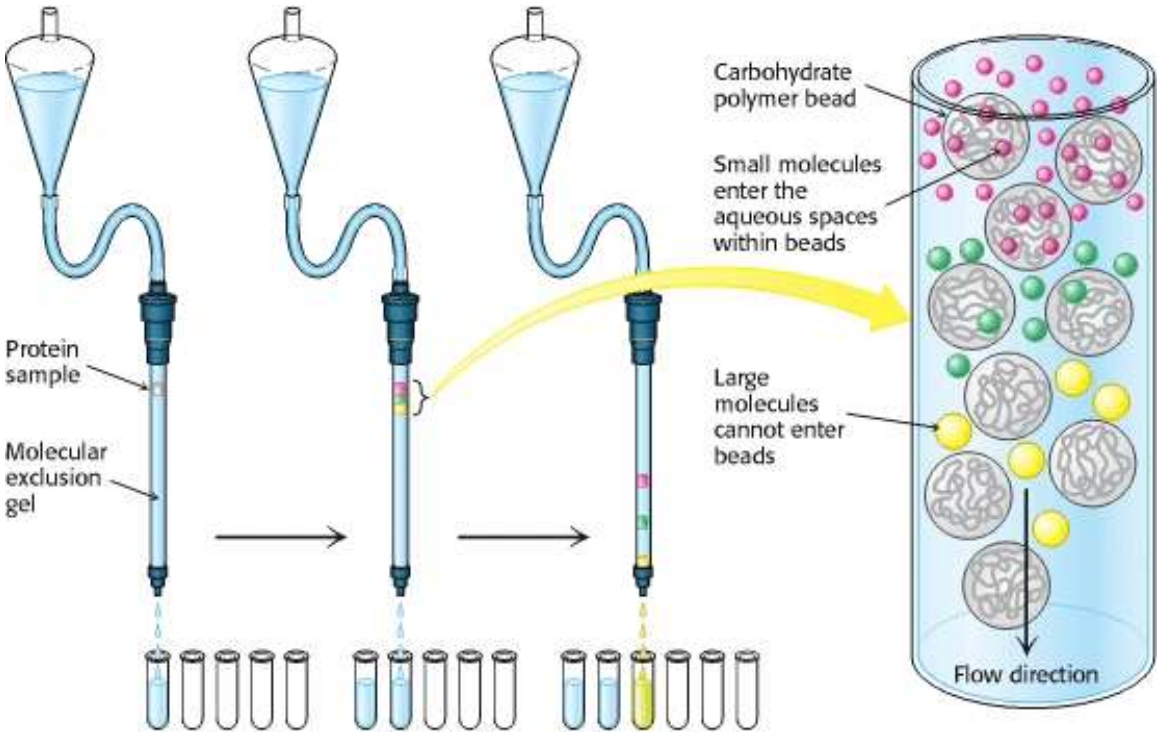


Figure 3.12. Gel Filtration Chromatography (Berg et al., 2002).

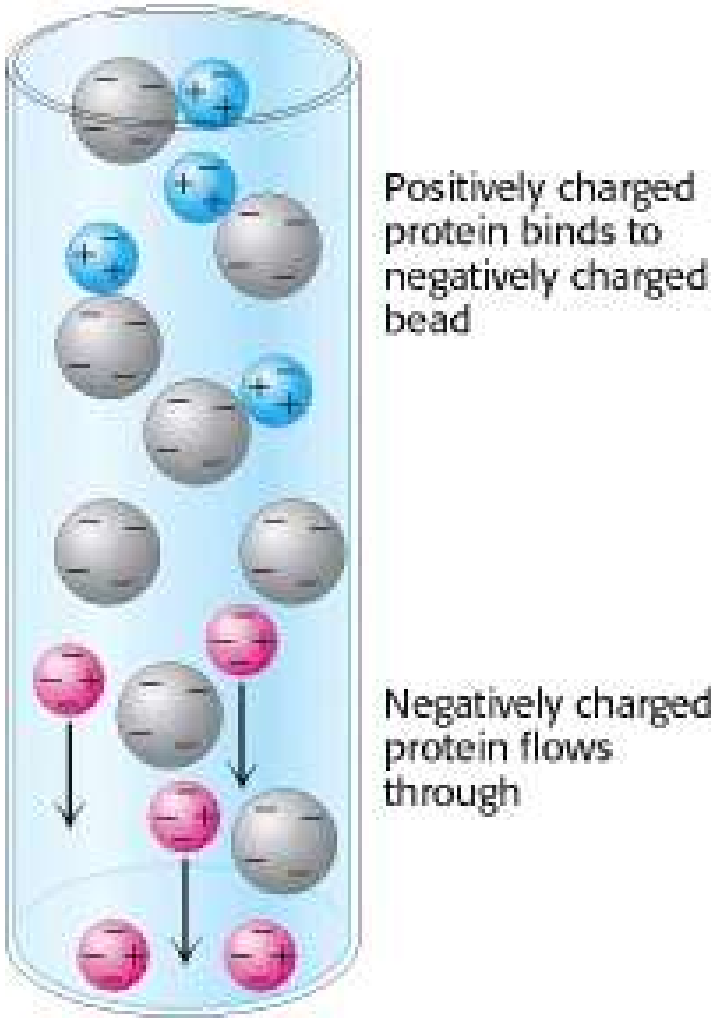


Figure 3.13. Ion-exchange chromatography (Berg et al., 2002)

Khan and Bano (2008) purified the cystatin obtained from goat lung using ion-exchange chromatography. The column used for ion-exchange chromatography was DEAE-cellulose column (2 X 7 cm) equilibrated with 50 mM sodium phosphate buffer (pH 8.0).

Sadaf et al. (2005) purified cystatins obtained from goat kidney using ion-exchange chromatography. DEAE cellulose column (2.1 X 7 cm) equilibrated with 0.05 M sodium phosphate buffer (pH 8.5).

3.9.3. Affinity Chromatography

Affinity chromatography separates proteins based on their affinity to specific chemical groups. A protein mixture is passed through a column packed with appropriate matrix (Lee and Lee, 2004). The high affinity proteins gets bonded to the matrix and retained. Affinity chromatography is highly powerful tool for the extraction of specific proteins (Urh et al., 2009; Berg et al., 2002). A schematic representation of affinity chromatography is shown in Figure 3.14.

Green et al. (1984) purified cystatin obtained from human liver sample using affinity chromatography. Cm-papain-Sepharose column (50 mm diameter, 300 mL bed volume) equilibrated with 50 mM- sodium phosphate buffer (pH 6.5), containing 0.5 M Nacl and 0.1 % Brij 35.

Trzisaka et al. (2006) purified egg white cystatin using affinity chromatography. The column used for chromatography was papain-Sepharose 4B column (7.5 X 5 cm) equilibrated with 10 mM Tris-Hcl (pH 7.5).

Matsuishi and Okitani (2002) purified cystatin obtained from rabbit skeletal muscle using affinity chromatography. Sephadex G-75 column equilibrated with 10 mM Tris-Hcl buffer (pH 7.2) containing 0.1 M Nacl and 1 mM NaN_3 was used.

3.10. Assay for Inhibitory Activity

The caseinolytic method can be used to determine the papain inhibitory activity. In this assay, casein is used as the protein substrate. The action of papain (enzyme) on

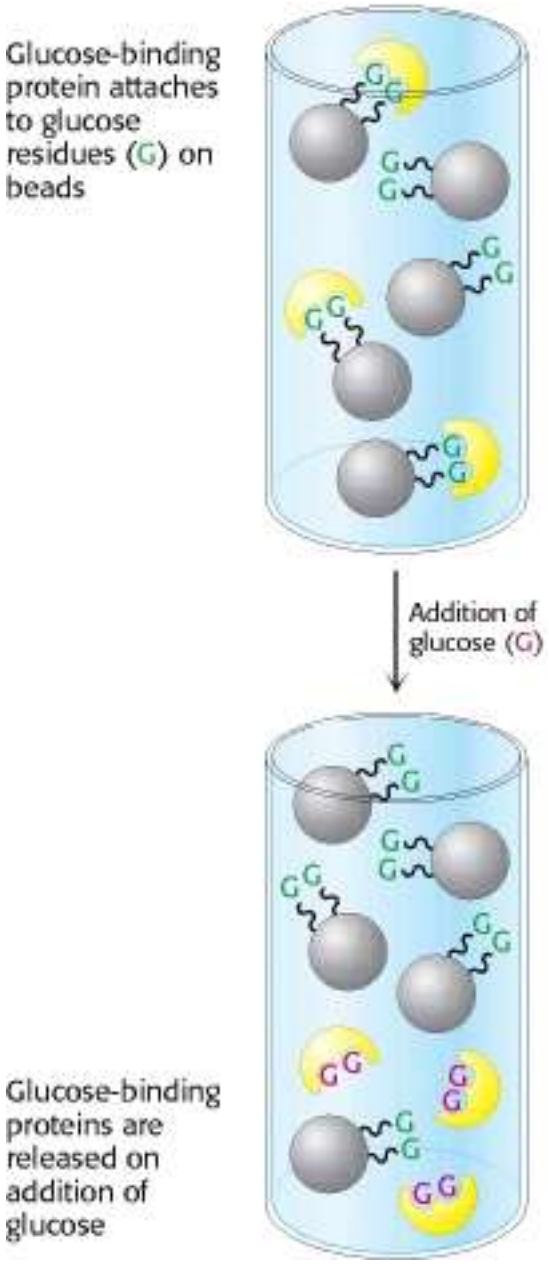


Figure 3.14. Affinity chromatography (Berg et al., 2002).

casein (protein substrate) forms TCA (tricholoro acetic acid) fractions and it is measured by the change in absorbance at 280 nm. Residual activity of papain in the presence of the inhibitor at 37 °C is measured as inhibitory activity (Murachi, 1970). Khan and Bano (2008) isolated and characterized cystatins form goat lung using the caseinolytic assay to determine the inhibitory activity of cystatin. Cystatins from goat kidney was isolated by Sadaf et al. (2005) and the cystatin inhibitory activity was monitored by the caseinolytic assay. Priyadarshini and Bano (2009) isolated cystatin from goat pancreas and they used the caseinolytic assay to determine cystatin inhibitory activity.

The procedure involved in the caseinolytic method is as follows. Papain solution is prepared by dissolving 6mg/ml of dry papain. 1 mL of papain is incubated with 1 mL purified inhibitor solution at 37°C for 10 minutes. After incubation, 2 m1 of 1% casein solution (w/v) is added to the solution and the mixture incubated for 10 min at 37°C, after which 2.5 mL of TCA (trichloroacetic acid) (5% w/v) is added to interrupt the reaction. The precipitate is removed by centrifugation and the absorbance of supernatant is measured at 280 nm. The TCA soluble peptide fraction of caesin formed by the action of papain in the presence and absence of inhibitor is then quantified using tyrosine as standard. The flow chart for the assay is shown in Figure 3.15 (Kunitz, 1947; Bijina, 2006).

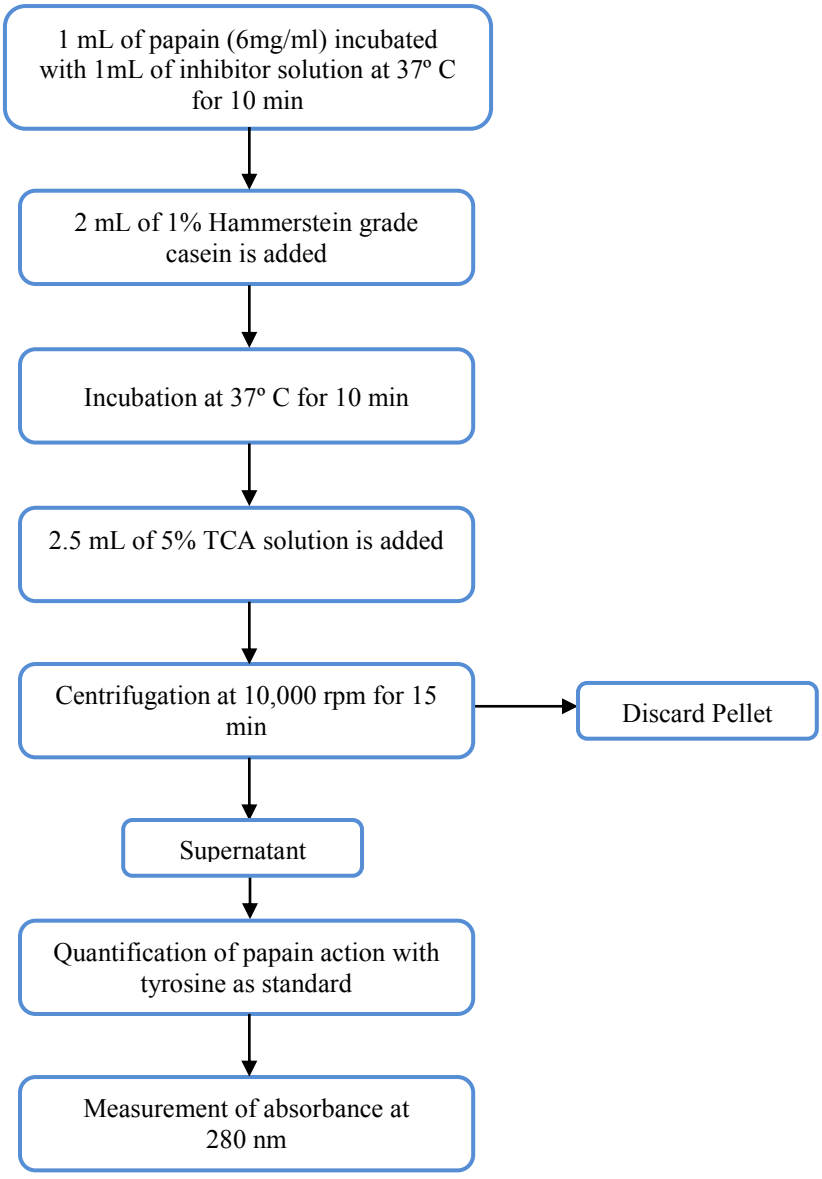


Figure 3.15. Assay for proteinase inhibitor activity (Kunitz, 1947; Bijina, 2006)

CHAPTER 4. EXPERIMENTAL MATERIALS

4.1. Bovine Samples

The bovine samples (lung and pancreas) and were obtained from Tuker slaughter house, Windsor, Nova Scotia. Frozen samples were collected in plastic containers and stored at -20˚C in Biotechnology Laboratory of the Process Engineering and Applied Sciences Department of Dalhousie University, Halifax, Nova Scotia.

4.2. Glassware

The glassware used for the experiment included pipettes, test tubes, conical flasks, separating funnels, beakers (500 mL, 1000mL). All glassware was washed with soap and tap water and rinsed with distilled water before use.

4.3. Chemicals and reagents

Trizma base, NaCl, ammonium sulphate, n- butyl alcohol, guanidine hydrochloride, casein, cysteine, 5,5'-Dithio-bis(2-Nitrobenzoic acid) (DTNB), DL-Di thiotheritol solution (DTT), BSA (bovine serum albumin), 500 mM Sodium carbonate solution, 0.6 N Trichloroacetic acid, papain (6mg/ml) were obtained from Sigma-Aldrich, Oakville, Ontario, Canada.

The reagents used in for the experiments included 1% NaCl buffer (0.003M EDTA, 10% (v/v %) 1-butanol, 1% NaCl), 0.1M potassium phosphate buffer, 0.1M phosphate buffer, sodium phosphate buffer. Tris- HCl was made by dissolving trizma buffer (tris base) in 1/3 volume of deionized water, Brilliant Blue G in phosphoric acid and methanol (500 mL), Folin & Ciocalteu's (F-C) Reagent, 1.1 mM Tyrosine Standard ,

4.4. Equipment

The equipment used in the experiments was: Precision water bath shaker (2870 Series, Thermo Scientific, Marietta, Ohio, USA), Precision water bath (280 Series ,Thermo Scientific, Marietta, Ohio, USA), rotary evaporator (Yamato RE540, Yamato Scientific America, Santa Clara, California, USA), homogenizer (Hamilton

Beach Model No. 53257, Southern Pines, North Carolina, USA), oven (Isotemp 655 F, Fisher Scientific, Cleveland, Ohio, USA), Centrifuge (Sorvall RT1, Thermo Scientific, Marietta, Ohio, USA), sonicator (Branson 2510, Branson Ultrasonics Corp, Danbury, Connecticut, USA), balance (Metler AE 200 and PM 4600 balance, Mettler-Toledo International Inc., Mississauga, Ontario, Canada), pH meter (Orion 5 Star, Thermo Scientific, Waltham, Massachusetts, USA), and spectrophometer (Genesys 10 S UV-VIS, Thermo Scientific, Marietta, Ohio, USA).

CHAPTER 5. EXPERIMENTAL PROCEDURE

5.1. Experimental Design

Cystatin (cysteine protease inhibitor) was extracted from three different samples: bovine lung, pancreatic tissue and mixture of lung and pancreas samples. The extraction procedure for cystatin recovery involved three steps: addition of buffer extraction, alkaline treatment and ammonium sulphate precipitation.

The study was carried out in three steps. In the first step, effects of incubation time (15, 30, 45 and 60 mins), pH (6.5, 7.0, 7.5, and 8.0) and ammonium sulphate saturation (25-45% and 45-60%) on total protein (TP), total activity (TA), specific activity (SA) and yield (Y) were studied. The experimental procedure is shown in Figure 5.1 and the experimental parameters are presented in Table 5.1. Three replicates were carried out which resulted in 288 runs.

In the second and the third steps, the bovine samples with the highest yields of cystatin were used to find optimal pH (3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5 and 11.5) and temperature (30, 40, 50, 60, 70, 80, and 90 ºC) for the maximal inhibitory assay. The experimental procedures for optimization of pH and temperature for maximal inhibitory activity are shown in Figure 5.2 and 5.3 and the experimental parameters are presented in Tables 5.1 and 5.3 respectively. Three replicates were carried out which resulted in 81 runs for the pH experiment and 63 runs for the temperature experiment.

5.2. Sample Preparation

The bovine lungs and pancreas samples were each first cut into small pieces (~ 5 cm^3). The pieces were weighed using a digital scale (Metler AE 200, Mettler-Toledo International Inc., Mississauga, Canada), marked and stored at -20°C for later use. All the samples were thawed at 4 °C prior to the extraction process.

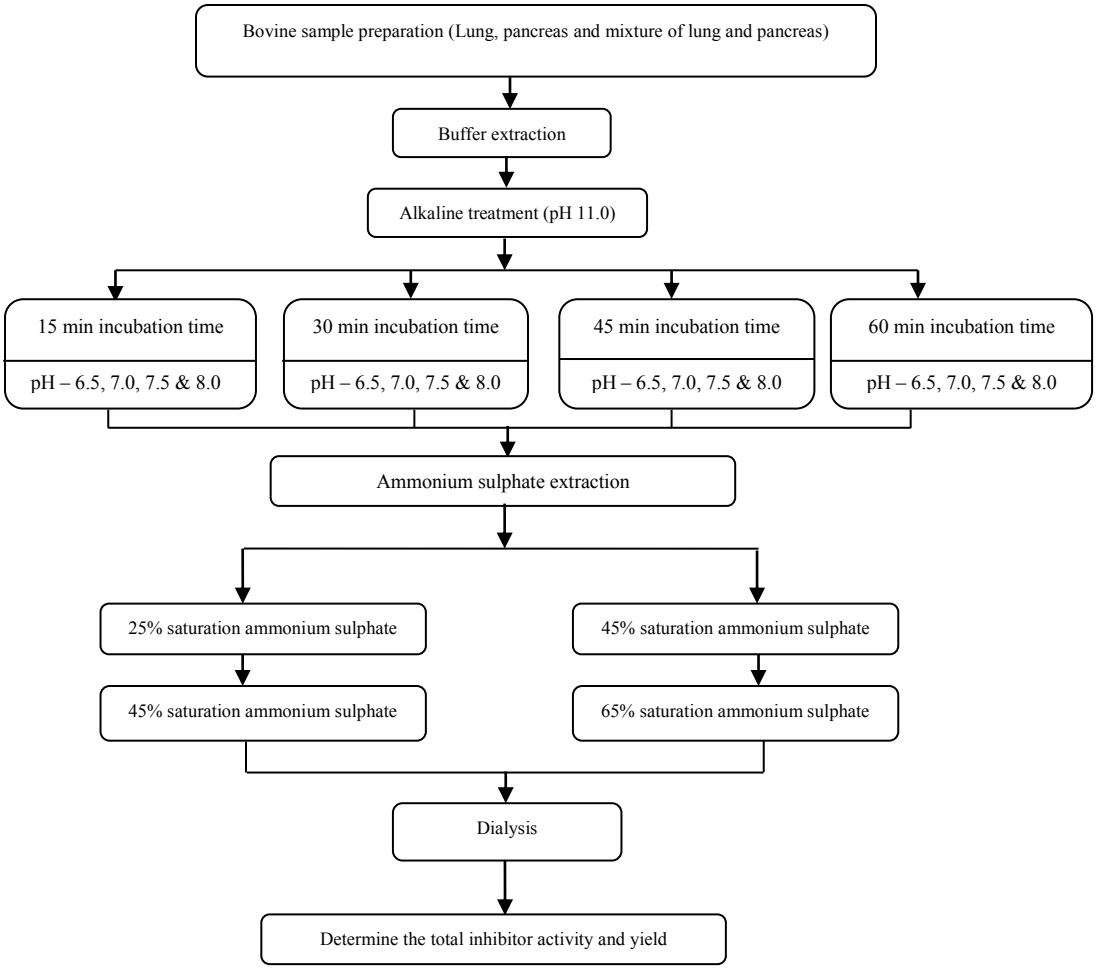


Figure 5.1. Experimental plan for the extraction of cystatin from bovine samples.

Table 5.1.Optimization of the ammonium sulphate extraction of cystatin for bovine samples

Factors	Parameters
pH (alkaline treatment)	11.0
Temperature	4°C
Bovine sample	Lung, pancreas and mixture of lung and pancreas
Incubation time	15 min, 30 min, 45 min, and 60 min
pH (auto activation)	6.5, 7.0, 7.5 and 8.0
Ammonium sulphate saturation	25-45%, 45%-60%

No. of replicates = 3
No. of runs = 288

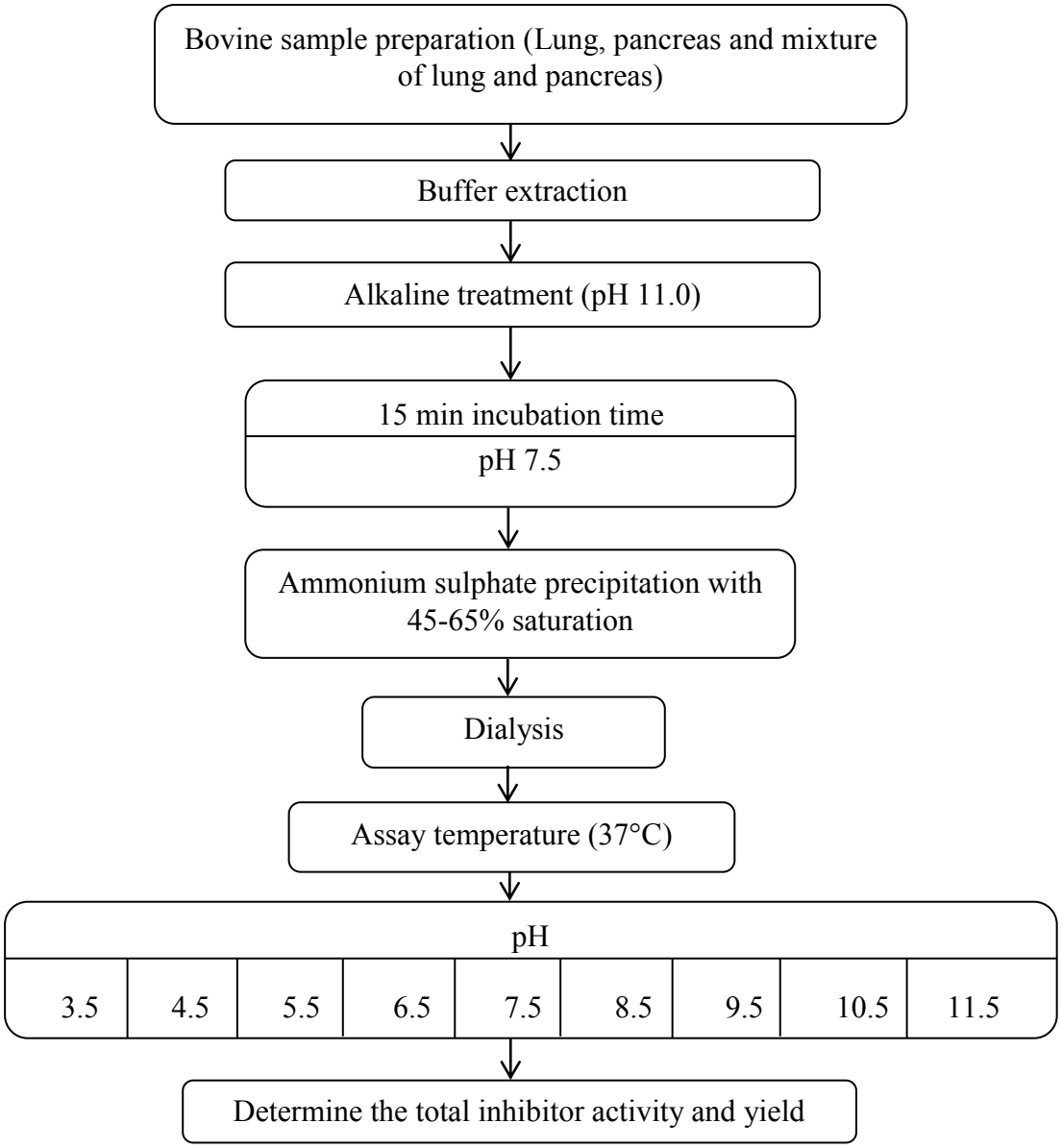


Figure 5.2. Experimental plan for the optimization of assay pH for maximal inhibitory activity cystatin from bovine samples.

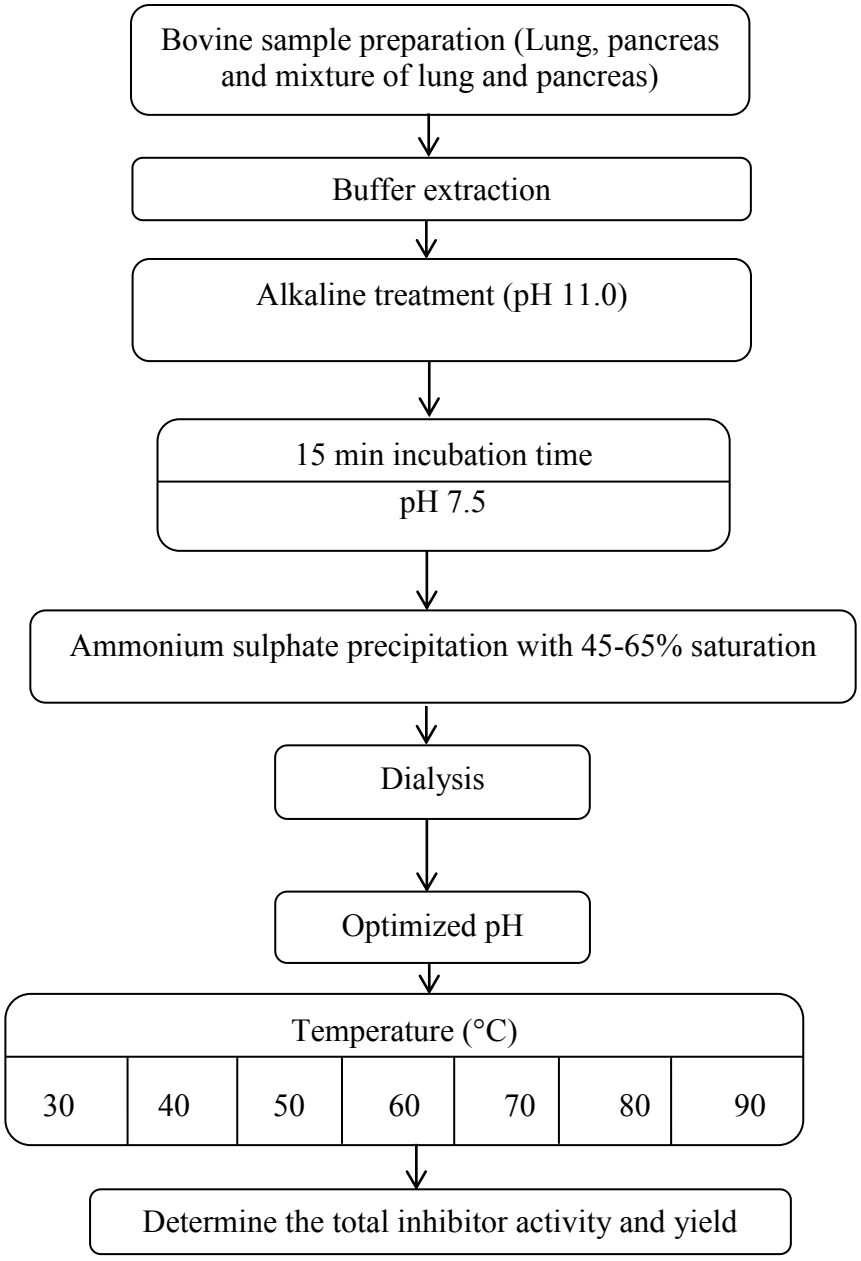


Figure 5.3. Experimental plan for the optimization of assay temperature (°C) for maximal inhibitory activity cystatin from bovine samples

Table 5.2. Optimization of pH for maximal inhibitory activity

Factors	Parameters
pH (alkaline treatment)	11.0
Temperature	4°C
Incubation time	30 min
pH (auto activation)	7.5
Bovine sample	Lung, pancreas and mixture of lung and pancreas
Ammonium sulphate saturation	45%-60%
pH (assay)	3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5 and 11.5

No. of replicates = 3
No. of runs = 81

Table 5.3. Optimization of temperature (ºC) for maximal inhibitory activity

Factors	Parameters
pH (alkaline treatment)	11.0
Temperature	4°C
Incubation time	30 min
pH (auto activation)	7.5
Bovine sample	Lung, pancreas and mixture of lung and pancreas
Ammonium sulphate saturation	45%-60%
Temperature (assay)	30, 40, 50, 60, 70, 80, and 90 ºC

No. of replicates = 3
No. of runs = 63

5.3. Extraction Procedure

The extraction was carried out according to the procedures described by Khan and Bano (2008) and Priyadarshini and Bano (2009). The extraction procedure involved three steps: Buffer extraction, alkaline treatment and ammonium sulphate precipitate. Experimental procedure used in the study is shown in Figure 5.4.

5.3.1. Buffer Extraction

The buffer extraction involved homogenizing and centrifugation of the sample. The buffer used had 1% NaCl, 3 mM EDTA, and 2 % n-butanol. A Hamilton Beach homogenizer (Model No. 53257, Southern Pines, and North Carolina) was used to homogenize 200 g (dry basis) of sample with 200 mL of buffer for 20 min. The homogenized sample was then centrifuged at 5000 rpm for 15 min at 4 °C in a refrigerated centrifuge (Sorvall RT1 Centrifuge, Thermo Scientific, Ohio, USA). The supernatant was collected and the pellet was discarded.

5.3.2. Alkaline Treatment

The supernatant was adjusted to pH 11 using a pH meter (Orion 5 Star pH meter, Thermo Scientific, Massachusetts, USA) and the addition of 3 M NaOH. The crude sample was then incubated for 15 min, 30 min, 45 min or 60 mins at 4 ºC. After incubation of sample, the pH was adjusted to the desired pH (6.5, 7.0, 7.5, or 8.0) with 2% (v/v) acetic acid.

5.3.3. Ammonium Sulphate Precipitate

After alkaline treatment, the samples (Lung, pancreas and mixture of lung and pancreas) were centrifuged (Sorvall RT1 Centrifuge, Thermo Scientific, Ohio, USA) at 4100 rpm for 50 min at room temperature (~20˚C). The pellet was discarded and the supernatant was collected for ammonium sulphate fractionation. The saturation levels were adjusted between 25-45%. The amounts of ammonium sulphate added to attain the desired saturations are tabulated in Table 5.4. For 25-45% saturation, the

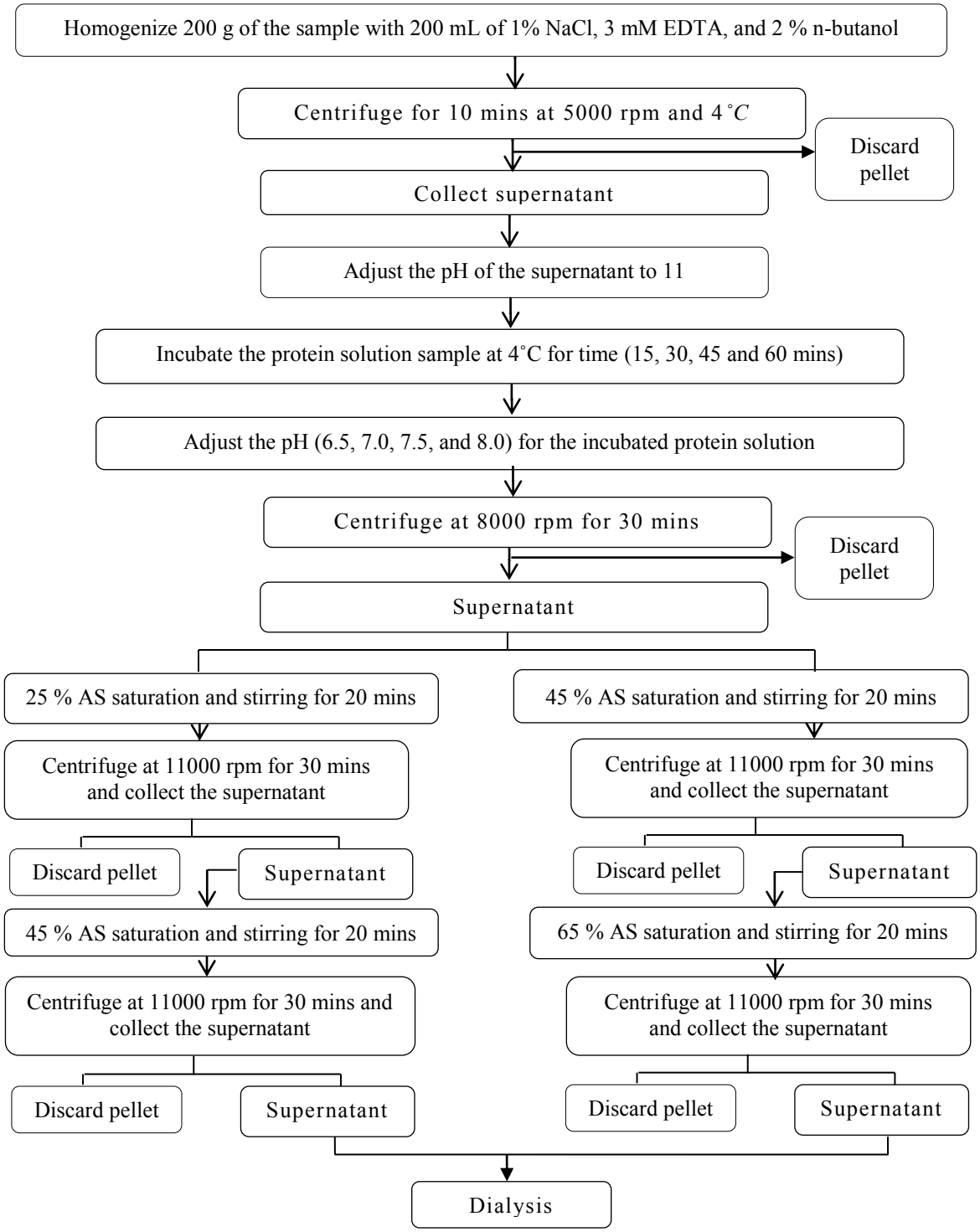


Figure 5.4. Extraction of cystatin from bovine lung sample, pancreas sample and mixture of both lungs and pancreas sample using the ammonium sulphate (AS) precipitation.

Table 5.4. Amount of ammonium sulphate required to attain the desired saturation (one liter of ammonium sulphate solution)

Desired Saturation (%)	Weight of $(NH_4)_2SO_4$ Required (g)
20	106
25	134
30	164
35	194
40	226
45	258
50	291
55	326
60	361
65	398
70	436
75	476
80	516
85	559
90	603
95	650
100	697

samples were first adjusted to 25% saturation by mixing with required amount of ammonium sulphate. They were then centrifuged (Sorvall RT1 Centrifuge, Thermo Scientific, Ohio, USA) at 4100 rpm for 30 min and the supernatant was collected. The saturation of the supernatant was then increased to 45 % by the addition of required ammonium sulphate and the mixture was again centrifuged. The supernatant was discarded and the pellet was dissolved in 50mM sodium phosphate buffer (pH 7.5). The fractions were obtained between 25% and 45 % ammonium sulphate saturation. The same extraction procedure was followed with 45-65% ammonium sulphate saturation were collected.

After the entire experimental procedure was carried out with all samples (Lung, pancreas and mixture of lung and pancreas), the fractions were dialyzed against the 50mm sodium phosphate buffer containing 1% NaCl. The collected fractions were tested for total inhibitory activity and yield with the protease inhibitor assay at each of the three steps involved in the extraction process.

5.4. Dialysis

After ammonium sulphate precipitation, the extract is then dialyzed. Dialysis tubing was used for the process. The tubing was placed in running distilled water for 15 mins before use. The tubing clip is used to seal one end of the tubing and the extracted is carefully transferred with a micropipette through the other end. The open end was sealed with another tubing clip. The tubing with the extract was suspended in 2% Nacl buffer (used during homogenization) in a beaker and placed at 4˚C. The buffer was changed every 6 hr interval. After 24 hr the salt precipitate was completely separated from the extract.

5.5. Inhibitory Activity Assay

The trypsin inhibitory activity by method of casein digestion (Kuntz, 1947) was used to determine the inhibitory activity of cystatin obtained from the bovine sample. Here, the ability of cystatin to inhibit the caseinolytic activity of papain is used to determine the inhibitory activity of cystatin. One unit of inhibitor activity is defined as the amount of

inhibitor causing a 0.001 absorbance change per minute. When casein is digested, tyrosine amino acid is liberated which is later compared a prepared standard tyrosine.

Casein (1g) was suspended in 100 mL of 0.1 M Sorensen's phosphate buffer (pH 7.6). The mixture was heated for 15 min in a boiling water bath. A complete solution of 1 % casein was formed. It was stored at 4˚C and was stable for a week. Papain solution was prepared by dissolving 6 mg/ml of dry papain. 0.5 mL of a suitable dilution of the extract containing protease inhibitor was added with 0.5 mL of papain and incubated at 37˚C for 15 min. Then 5 mL of the prepared 1% casein solution was added to the above mixture. It was again incubated at 37˚C for 30 min. Termination of the reaction was achieved by the addition of 5 mL of 0.44 M TCA. The mixture was centrifuged at 10,000 rpm for 15 min. The pellet was discarded and the absorbance of the supernatant was measured at 280 nm in a UV spectrophotometer against tyrosine standard prepared earlier. Protease Colorimetric Detection Kit (Product Code PC0100, Sigma Aldrich, Ottawa, Canada) was used for quantification. One unit of papain activity was defined as the amount of enzyme that liberated 1 µg of tyrosine per milliliter of the reaction mixture per minute under the assay conditions. One unit of inhibitor activity was defined as the decrease by one unit of absorbance of TCA soluble casein hydrolysis product liberated by papain action at 280 nm per minute at 37° C in the given assay volume.

5.6. Experimental Analysis

5.6.1. Total Protein Content (TP)

The TP was determined by calculating the protein concentration (Cp). The standard Bradford assay method was used to determine the protein concentration (Bradford, 1976). The assay works on the principle of complex formation between the Brilliant Blue G dye and the proteins in the sample solution. BSA (bovine serum albumin) was used as the protein standard. The assay was performed at room temperature. The absorbance range at which the standard and the samples are compared was 595 nm. Standard 3.1 mL assay protocol was followed as described below

The concentration ranges of BSA used were between 0.1 - 1.0 mg/ mL. The concentrations BSA are shown in Table 5.5. The Bradford reagent was kept at room temperature prior to use. 250mL of BSA standard was prepared by dissolving 500 mg of BSA in distilled water. 0.1 mL of each standard was mixed with 3 mL of Bradford reagent. Each standard was measured at 595nm against the blank which had 7 mL of buffer solution (distilled water).

The results were compared with the standard in order to determine the protein concentration of the sample protein. The absorbance readings measured at 595nm for different protein concentrations are shown in Table 5.6. The standard curve produced by the assay data is represented in Figure 5.5. The total protein content (TP) was calculated as follows:

$$TP = Cp \ X \ TV \tag{5.1}$$

Where:

TP = Total protein content (mg)

C_p = Protein concentration (mg/mL)

TV = Total volume (mL)

5.6.2. Total Inhibitor Activity (TA)

The total inhibitory activity (TA) of cystatin was calculated by finding the inhibitory activity. µmoles of tyrosine liberated was found by using tyrosine as standard. The inhibitor activity (IA) of cystatin on papain in the presence of the inhibitor was expressed in terms of units/mL and it was calculated according to Bijina (2006)

$$IA = \frac{\mu mole\ of\ Tyrosine\ released\ X\ (11)}{(2)\ X\ (10)\ X\ (1)} \tag{5.2}$$

Table 5.5. The BSA concentrations used in the assay to make standard curves.

[BSA] Protein standard (mg/mL)	Volume (mL) of 2mg/mL of BSA stock	Volume (mL) of distilled water
0.0	0.0	10.0
0.1	0.5	9.5
0.2	1.0	9.0
0.3	1.5	8.5
0.4	2.0	8.0
0.5	2.5	7.5
0.6	3.0	7.0
0.7	3.5	6.5
0.8	4.0	6.0
0.9	4.5	5.5
0.10	5.0	5.0

Table 5.6. Absorbance measured at 595 nm for the BSA protein concentrations.

	BSA Concentration (mg/mL)										
Sample	Blank	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
1	0	0.031	0.0536	0.0639	0.136	0.159	0.215	0.169	0.241	0.275	0.293
2	0	0.028	0.0528	0.065	0.149	0.146	0.225	0.194	0.264	0.296	0.262
3	0	0.033	0.0521	0.060	0.130	0.162	0.219	0.185	0.261	0.269	0.299
Average	0	0.031	0.0521	0.062	0.138	0.155	0.219	0.182	0.255	0.280	0.284

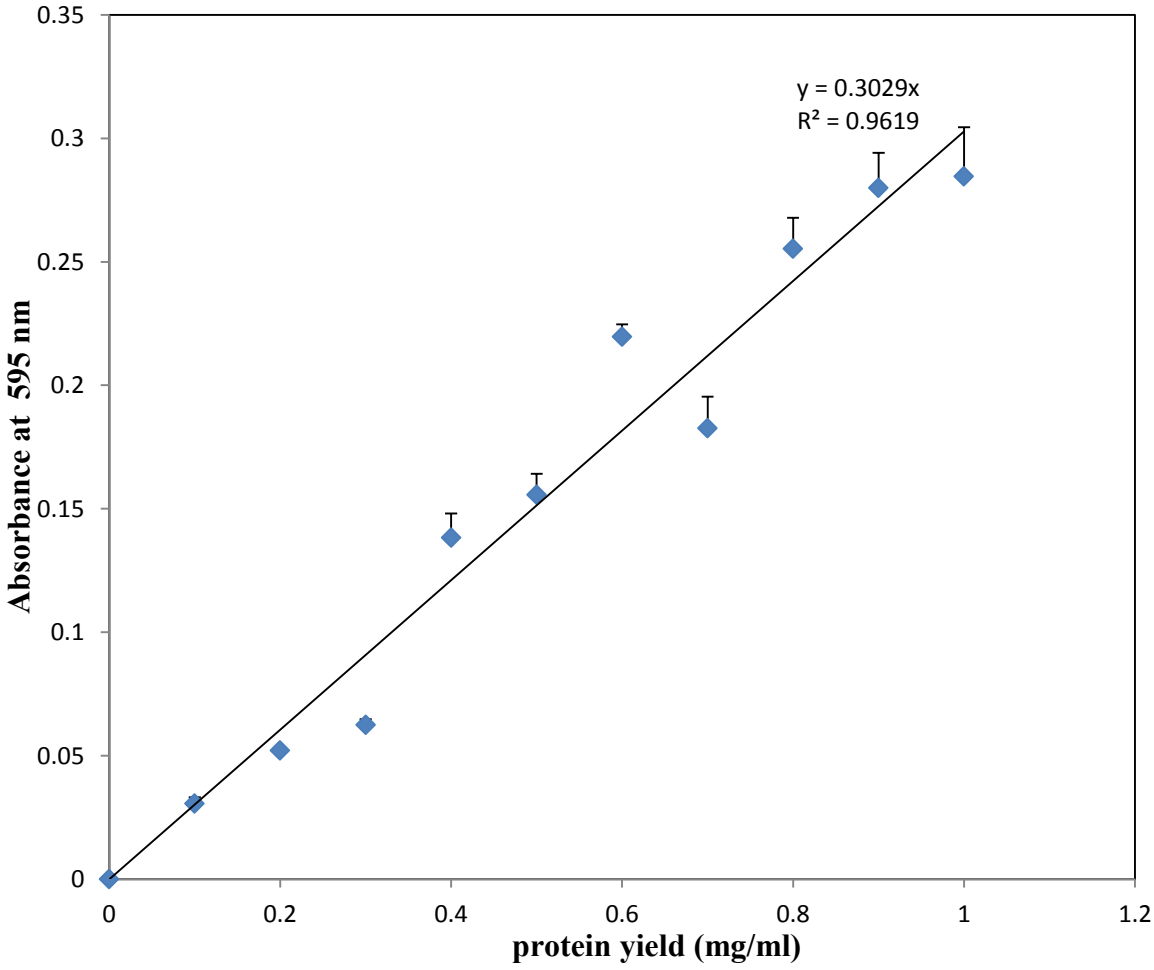


Figure 5.5. Standard curve for protein concentration (mean±std, n=3).

Where:

IA = Inhibitor activity (units/mL, where units in OD mL^{-1} min^{-1})

µmoles of tyrosine = OD (from standard)

11 = Total volume of assay (mL)

2 = volume of enzyme used in assay

10 = Time of assay (mins)

1 = Volume used in spectrophotometric detection (mL)

The total inhibitory activity (TA) was expressed was calculated according to Bijina (2006):

$$TA = IA \ X \ TV \qquad (5.3)$$

Where:

TA = Total inhibitory activity (units in OD mL^{-1} min^{-1})

IA = Inhibitory activity (units/mL)

TV = Total volume of the sample (mL)

5.6.3. Inhibitory Activity Percentage (I)

The inhibitory activity percentage was expressed in % and calculated as follows:

$$I = \frac{(\text{Amount of tyrosine released without inhibitor} - \text{Amount of tyrosine released with inhibitor})}{(\text{Amount of tyrosine released without inhibitor})} \ X\ 100 \qquad (5.4)$$

5.64. Specific Activity (SA)

The specific activity of the sample was calculated by dividing the total inhibitory activity (TA) by the total protein content (TP) and was expressed as Units / mg.

$$SA = TA / TP \qquad (5.5)$$

Where:

SA = specific activity (units/mg)

TA = Total inhibitory activity (units)

TP = Total protein content (mg)

5.6.5. Yield (Y)

Yield is evaluated by dividing the total activity of extracted sample and the total activity of the crude sample

$$\text{Y}\ (\%) = \frac{\text{TA of extracted sample}}{\text{TA of crude sample}}\ \text{X}\ 100 \qquad (5.6)$$

Where:

TA = Total inhibitory activity (mg)

5. 7. Statistical Analysis

The protein concentration was found and the recovery yield, specific activity and standard errors were calculated. Analyses of variance (ANOVA) were performed on all the statistical data using Minitab Statistics Software (Version 16.2.3, Minitab Inc). The TUKEY test was also performed on the data.

CHAPTER 6. RESULTS

6.1. Crude Extraction

The crude extraction was performed on the bovine lung sample, pancreas sample and mixture of lung and pancreas sample. The crude extraction included: (a) homogenization of the crude sample, (b) centrifugation for 10 mins at 5000 rpm and 4ºC and (c) adjusting the pH of the supernatant to 11 (alkaline treatment). The total proteins content (TP), total inhibitory activity (TA), specific activity (SA) and yield (Y) were then determined.

6.1.1. Volume (V)

The volume results are shown in Table 6.1. After centrifugation and alkaline treatment, the volume (V) was reduced from 400 to 385 mL (5%), from 400 to 360 mL (13.33%) and from 400 to 370 mL (10%) for the bovine lung, pancreas and mixture of lung and pancreas samples, respectively.

Analysis of variance was performed on the data using Minitab (Version 16.2.3, Minitab Inc, State College, Pennsylvania, USA).The results are shown in Table 6.2. The effects of sample type and steps (crude and alkaline) were significant at 0.001 level. There was also a significant interaction between the parameters at the 0.001 level.

Tukey grouping was also performed on the data as shown in Table 6.3. The bovine lung, pancreas and mixture of lung and pancreas samples were significantly different from each other at the 0.05 level. The highest average mean volume (V) of 392.5 mL was obtained from the bovine lung sample. The two steps (crude and alkaline) were significantly different from each other at the 0.05 level. The highest average mean volume (V) of 400 mL was obtained from the crude step.

6.1.2. Total Protein (TP)

The total protein (TP) results are shown in Table 6.1. After centrifugation and alkaline treatment, the total protein (TP) was reduced from 10655 to 7569 mg (28%), from 13786 to 10242 mg (25.7%), from 20356 to 13835 mg (32.03%) for the bovine lung, pancreas and mixture of lung and pancreas samples, respectively.

Table.6.1. Crude extraction of cystatin from lung, pancreas and mixture of lung and pancreas

Sample	Step	Volume (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
I (lung sample)	Crude^{+}	400±0.00	10655±0.63	842.9±0.39	0.087±0.008	100.00
	Alkaline treatment (pH 11)$^{++}$	385±0.98	7569±0.93	760.9±0.45	0.100±0.008	90.30±0.15
II (pancreas sample)	Crude $^{+}$	400±0.00	13786±0.54	75.3±0.15	0.005±0.008	100.00
	Alkaline treatment (pH 11) $^{++}$	360±0.98	10242±0.78	60.3±0.36	0.006±0.003	79.90±0.42
III (lung and pancreas sample)	Crude $^{+}$	400±0.00	20356±0.43	297.9±0.33	0.017±0.000	100.00
	Alkaline treatment (pH 11) $^{++}$	370±0.74	13835±0.47	246.6±0.46	0.018±0.000	82.79±0.28

+ : After homogenization
++ : After adjusting the pH to 11
Sample volume = 200g
Replicates = 3
TP : Total protein content
TA : Total inhibitory activity
SA : Specific activity
Y : Yield

Table 6.2. Analysis of variance for volume (V) - crude extraction

Source	DF	SS	MS	F	P
Total	17	4912.50			
Model					
ST	2	475.00	237.50	8.14	0.0050
STEP	1	3612.50	3612.50	123.86	0.0001
ST*STEP	2	475.00	237.50	8.14	0.0050
Error	12	350.00	29.17		

ST :Sample type
DF :Degree of freedom
SS :Sum of square
MS :Mean of square
R^2 :0.891
CV: 4.0%

Table 6.3. Tukey grouping for volume (V) - crude extraction

Factors	Level	N	Mean V (mL)	Tukey Grouping
Sample Type	Lung sample	6	392.5	A
	Lung and pancreas sample	6	385.0	B
	Pancreas sample	6	380.0	C
Step	Crude	9	400.0	A
	Alkaline treatment	9	371.7	B

Means that share the same letter are not significantly different from one another at the 0.05 level.

Analysis of variance was performed on the data using Minitab (Version 16.2.3, Minitab Inc, City State, Pennsylvania, USA). The results are shown in Table 6.4. The effects of sample type and steps (crude and alkaline) were significant at the 0.001 level. There was also a significant interaction between the parameters at the 0.001 level.

Tukey grouping was also performed on the data as shown in Table 6.5. The bovine lung, pancreas and mixture of lung and pancreas samples were significantly different from each other at the 0.05 level. The highest average mean total protein (TP) of 17096 mg was obtained from the mixture of lung and pancreas sample. The two steps (crude and alkaline) were significantly different from each other at the 0.05 level. The highest average mean total protein (TP) of 14932 mg was obtained from the crude step.

6.1.3. Total Activity (TA)

The total activity results are shown in Table 6.1. After centrifugation and alkaline treatment, the total inhibitory activity (TA) was reduced from 842.9 to 760.9 units (9.72%), from 75.3 to 60.3 units and from 297.9 to 246.6 units (17.2%) for the bovine lung, pancreas and mixture of lung and pancreas samples, respectively.

Analysis of variance was performed on the data using Minitab (Version 16.2.3, Minitab Inc, State College, Pennsylvania, USA). The analysis of variance results are shown in Table 6.6. The effects of sample type and step (crude and alkaline) were significant at the 0.001 level. There was also a significant interaction between the parameters at the 0.001 level.

Tukey grouping was also performed on the data as shown in Table 6.7. The bovine lung, pancreas and mixture of lung and pancreas samples were significantly different from each other at the 0.05 level. The highest average mean total activity (TA) of 885.75 Units was obtained from the bovine lung sample. The two steps (crude and alkaline) were significantly different from each other at the 0.05 level. The highest average mean total activity (TA) of 460.73 Units was obtained from the crude step.

6.1.4. Specific Activity (SA)

The specific activity (SA) results are shown in Table 6.1. After centrifugation and

Table 6.4. Analysis of variance for total protein (TP) - crude extraction

Source	DF	SS	MS	F	P
Total	17	292869439			
Model					
ST	2	195959037	97979519	4858488.52	0.0001
STEP	1	86474401	86474401	4287986.80	0.0001
ST*STEP	2	10435759	10435759	5217879.00	0.0001
Error	12	242	20		

ST :Sample type
DF :Degree of freedom
SS :Sum of square
MS :Mean of square
R^2 :1.00
CV: 3.2%

Table 6.5. Tukey grouping for total protein (TP) - crude extraction

Factors	Level	N	Mean TP (mg)	Tukey Grouping
Sample Type	Lung and pancreas sample	6	17096	A
	pancreas sample	6	12014	B
	Lung sample	6	9112	C
Step	Crude	9	14932	A
	Alkaline treatment	9	10549	B

Means that share the same letter are not significantly different from one another at the 0.05 level.

Table 6.6. Analysis of variance for total activity (TA) - crude extraction

Source	DF	SS	MS	F	P
Total	17	2040788			
Model					
ST	2	2023483	1011742	445047.60	0.0001
STEP	1	13795	13795	6068.01	0.0001
ST*STEP	2	3483	1741	766.04	0.0001
Error	12	27	2		

ST :Sample type
DF :Degree of freedom
SS :Sum of square
MS :Mean of square
R^2 :1.00
CV :8.0%

Table 6.7. Tukey grouping for total activity (TA) - crude extraction

Factors	Level	N	Mean TA (Units)	Grouping
Sample Type	Lung sample	6	885.75	A
	Lung and pancreas sample	6	328.85	B
	Pancreas sample	6	84.55	C
Step	Crude	9	460.73	A
	Alkaline treatment	9	405.37	B

Means that share the same letter are not significantly different from one another at the 0.05 level.

alkaline treatment, the specific activity (SA) showed an increase from 0.087 to 0.100 Units/mg (27.5%), 0.005 to 0.006 Units/mg (16.66%) and from 0.015 to 0.018 Units/mg (5.88%) for bovine lung, pancreas and mixture of lung and pancreas samples, respectively.

Analysis of variance was performed on the data using Minitab (Version 16.2.3, Minitab Inc, State College, Pennsylvania, USA). The results are shown in Table 6.8. The effects of sample type and steps (crude and alkaline) were significant at the 0.001 level. There was also a significant interaction between the parameters at the 0.001 level.

Tukey grouping was also performed on the data as shown in Table 6.9. The bovine lung, pancreas and mixture of lung and pancreas samples were significantly different from each other at the 0.05 level. The highest average mean specific activity (SA) of 0.043233Units/mg was obtained from the bovine lung sample. The two steps (crude and alkaline) were significantly different from each other at the 0.05 level. The highest average mean specific activity (SA) of 0.099000 Units/mg was obtained from the crude step.

6.1.5. Yield (Y)

The yield (Y) results are shown in Table 6.1. After centrifugation and alkaline treatment, the yield (Y) was 90.30%, 79.90% and 82.79 % for bovine lung, pancreas and mixture of lung and pancreas samples, respectively.

Analysis of variance was performed on the data using Minitab (Version 16.2.3, Minitab Inc, State College, Pennsylvania, USA). The results are shown in Table 6.10. The effects of sample type and steps (crude and alkaline) were significant at the 0.001 level. There was also a significant interaction between the parameters at the 0.001 level.

Tukey grouping was also performed on the data as shown in Table 6.11. The bovine ung, pancreas and mixture of lung and pancreas samples were significantly different from each other at the 0.05 level. The highest average mean yield (Y) of 95.39% was obtained from bovine lung sample.

Table 6.8. Analysis of variance for specific activity (SA) - crude extraction

Source	DF	SS	MS	F	P
Total	17	0.0340656			
Model					
ST	2	0.0331978	0.0165989	88190.36	0.0001
STEP	1	0.0003150	0.0003150	1673.63	0.0001
ST*STEP	2	0.0005505	0.0002753	1462.44	0.0001
Error	12	0.0000023	0.0000002		

ST :Sample type
DF :Degree of freedom
SS :Sum of square
MS :Mean of square
R^2 :1.00
CV :11.4%

Table 6.9. Tukey grouping for specific activity (SA) - crude extraction

Factors	Level	N	Mean SA (Units/mg)	Tukey Grouping
Sample Type	Lung sample	6	0.099000	A
	Lung and pancreas sample	6	0.017500	B
	Pancreas sample	6	0.000650	C
Step	Alkaline treatment	9	0.043233	A
	Crude	9	0.034867	B

Means that share the same letter are not significantly different from one another at the 0.05 level..

Table 6.10. Analysis of variance for yield (Y) - crude extraction

Source	DF	SS	MS	F	P
Total	17	1246.14			
Model					
ST	2	90.14	45.07	23721.16	0.0001
STEP	1	1065.83	1065.83	560965.50	0.0001
ST*STEP	2	90.14	45.07	23721.16	0.0001
Error	12	0.02	0.00		

ST :Sample type
DF :Degree of freedom
SS :Sum of square
MS :Mean of square
R^2 :1.00
CV :9.0%

Table 6.11. Tukey grouping for yield (Y) - crude extraction

Factors	Level	N	Mean Y (%)	Tukey Grouping
Sample Type	Lung sample	6	95.39	A
	Lung and pancreas sample	6	91.40	B
	Pancreas sample	6	90.14	C
Step	Crude	9	100.00	A
	Alkaline treatment	9	84.61	B

Means that share the same letter are not significantly different at the 0.05 level

The two steps (crude and alkaline) were significantly different from each other at the 0.05 level. The highest average mean yield (Y) of 100% was obtained from the crude step.

6.2. Ammonium sulphate Extraction

The ammonium sulphate extraction method was used for the extraction of cystatin from the bovine lung, pancreas and mixture of lung and pancreas samples. The extraction process was performed with three materials at different pH levels (6.5, 7.0, 7.5, and 8.0), incubation times (15, 30, 45 and 60) and ammonium sulphate saturation levels (25-45% and 45-65%). The results are shown in Tables 6.12 - 6.14.

6.2.1. Total Protein (TP)

The effects of sample types (bovine lung, pancreas and mixture of lung and pancreas), pH (6.5, 7.0, 7.5, and 8.0), incubation time (15, 30, 45 and 60 min) and ammonium sulphate saturation level (25-45% and 45-65%) on the total protein (TP) of cystatin are shown in Tables 6.12 - 6.14. The total protein (TP) of cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas varied from 2251.0 to 1458.8 mg, from 1855.9 to 1292.0 mg and 1957.3 to 1407.8 mg, respectively.

Analysis of variance was performed on the total protein data using Minitab (Version 16.2.3, Minitab Inc, State College, Pennsylvania, USA). The results are shown in Table 6.15. The effects of sample type, pH, incubation time, ammonium sulphate saturation were significant at the 0.001 level. The two, three and four way interactions between the parameters were also significant at the 0.001 level.

The Tukey grouping was also performed on the total protein (TP) data as shown in Table 6.16. The bovine lung, pancreas and mixture of lung and pancreas samples were significantly different from each other at the 0.05 level. The highest average total protein (TP) mean of 2090 mg was obtained from the bovine lung sample. All the incubation times were significantly different from each other at the 0.05 level. The highest average total protein (TP) mean of 2277 mg was obtained with the 15 min incubation. All pH levels were significantly different from each other at the 0.05 level. The highest average

Table 6.12. Effects of incubation times, pH and ammonium sulphate saturation levels on TP, TA, SA and Y of cystatin extracted from bovine lung.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
15	6.5	25-45%	210.5±0.38	2251.0±0.33	203.0±0.54	0.090±0.011	24.08±0.19
		45-65%	210.0±0.00	2557.0±0.43	253.0±0.56	0.099±0.010	30.01±0.19
	7.0	25-45%	210.0±0.00	2677.3±0.52	265.2±0.60	0.099±0.011	31.43±0.21
		45-65%	208.0±0.31	2964.0±0.66	386.9±0.29	0.131±0.004	45.79±0.10
	7.5	25-45%	210.5±0.31	2756.7±0.41	370.7±0.30	0.134±0.006	43.97±0.10
		45-65%	209.5±0.21	3036.3±5.90	450.1±0.17	0.148±0.003	53.39±0.10
	8.0	25-45%	210.5±0.55	1890.3±0.85	156.3±0.33	0.083±0.005	18.54±0.11
		45-65%	210.5±0.60	2249.7±1.85	202.3±0.55	0.090±0.006	24.00±0.05
30	6.5	25-45%	212.0±0.23	2069.7±0.71	276.4±0.87	0.134±0.018	32.79±0.30
		45-65%	210.5±0.38	2165.0±0.69	295.4±0.54	0.140±0.001	35.04±0.19
	7.0	25-45%	210.0±0.31	2199.7±7.33	311.8±1.76	0.142±0.005	36.99±1.90
		45-65%	210.0±0.00	2561.3±0.35	424.8±0.45	0.166±0.009	50.39±0.16
	7.5	25-45%	212.0±0.40	2549.2±0.34	439.4±0.38	0.163±0.008	52.12±0.13
		45-65%	210.5±0.31	2788.7±0.57	505.8±0.47	0.172±0.010	60.07±0.17
	8.0	25-45%	210.5±0.31	1715.1±0.73	188.7±0.56	0.118±0.010	22.38±0.06
		45-65%	210.0±0.58	1930.0±0.69	237.0±0.54	0.130±0.011	28.11±0.19

Replicates = 3
V : Volume
TP : Total protein content
TA : Total inhibitory activity
SA : Specific activity
Y : Yield

Table 6.12. Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
45	6.5	25-45%	210.0±0.23	1790.0±0.76	239.3±0.85	0.122±0.019	23.35±0.30
		45-65%	210.9±0.23	1986.7±0.60	251.6±0.30	0.133±0.006	29.84±0.11
	7.0	25-45%	210.0±0.58	1960.0±2.50	274.3±3.13	0.136±0.015	30.54±0.35
		45-65%	210.5±0.38	2096.7±0.38	291.4±0.50	0.154±0.003	34.57±0.05
	7.5	25-45%	210.5±0.31	2055.3±0.67	310.4±1.79	310.4±1.79	310.4±1.79
		45-65%	212.0±0.40	2213.0±0.52	329.0±0.60	0.156±0.046	39.03±0.21
	8.0	25-45%	210.0±0.58	1609.7±0.80	179.8±0.49	0.108±0.0012	21.33±0.35
		45-65%	210.0±0.31	1654.1±3.68	207.1±6.40	0.125±0.0035	23.56±0.68
60	6.5	25-45%	212.0±0.23	1558.3±0.80	154.2±1.15	0.099±0.0009	18.29±0.07
		45-65%	210.5±0.38	1651.6±2.33	184.6±0.72	0.119±0.0006	21.90±0.05
	7.0	25-45%	210.0±0.31	1655.7±2.03	202.9±0.46	0.123±0.0003	24.07±0.08
		45-65%	210.0±0.00	1819.7±0.54	248.4±0.77	0.144±0.0003	29.46±0.07
	7.5	25-45%	210.5±0.31	1717.2±1.14	269.0±0.64	0.157±0.0015	31.91±0.14
		45-65%	209.5±0.21	1986.9±0.44	295.0±0.50	0.148±0.012	34.99±0.17
	8.0	25-45%	210.5±0.55	1418.4±1.13	144.0±0.15	0.102±0.003	16.08±0.02
		45-65%	210.0±0.31	1458.8±0.33	164.0±0.45	0.112±0.012	17.22±0.16

Replicates = 3

V : Volume

TP : Total protein content

TA : Total inhibitory activity

SA : Specific activity

Y : Yield

Table 6.13. Effects of incubation times, pH and ammonium sulphate saturation levels on TP, TA, SA and Y of cystatin extracted from bovine pancreas.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
15	6.5	25-45%	175.5±0.38	1855.9±0.45	14.4±0.05	0.007±0.000	19.12±0.06
		45-65%	175.0±0.35	1956.0±0.47	17.2±0.18	0.009±0.004	22.84±0.08
	7.0	25-45%	178.0±0.23	1953.3±0.26	18.4±0.48	0.010±0.011	24.47±0.17
		45-65%	175.0±0.00	2100.3±0.22	22.8±0.32	0.011±0.007	30.31±0.38
	7.5	25-45%	175.0±0.00	2199.3±0.33	26.7±0.30	0.011±0.006	35.45±0.35
		45-65%	175.5±0.23	2573.7±0.48	28.9±0.24	0.012±0.005	38.37±0.29
	8.0	25-45%	175.5±0.15	1628.7±0.40	8.0±0.29	0.006±0.000	10.67±0.34
		45-65%	175.5±0.11	1673.3±0.64	8.2±0.22	0.008±0.005	10.90±0.26
30	6.5	25-45%	175.0±0.35	1743.2±0.62	16.4±0.35	0.009±0.008	21.73±0.41
		45-65%	175.5±0.15	1758.7±0.34	19.6±0.34	0.012±0.008	25.57±0.40
	7.0	25-45%	175.5±0.23	1887.8±0.45	20.2±0.55	0.011±0.009	26.73±0.47
		45-65%	175.0±0.35	1986.3±0.59	24.2±0.55	0.013±0.012	32.01±0.64
	7.5	25-45%	175.5±0.23	2054.1±0.52	27.8±0.31	0.014±0.07	36.93±0.36
		45-65%	175.0±0.35	2214.7±0.40	29.8±0.65	0.016±0.01	39.57±0.76
	8.0	25-45%	175.5±0.15	1529.1±0.54	9.7±0.19	0.010±0.006	12.91±0.29
		45-65%	175.0±0.00	1634.4±0.71	13.2±0.45	0.008±0.011	17.49±0.53

Replicates = 3

V : Volume
TP : Total protein content
TA : Total inhibitory activity
SA : Specific activity
Y : Yield

Table 6.13. Continued.

Incubation Time (min)	pH	Ammonium Sulphate saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
45	6.5	25-45%	175.5±0.38	1453.0±0.33	13.60±0.26	0.008±0.007	18.06±0.30
		45-65%	175.0±0.35	1615.7±2.60	15.13±0.37	0.010±0.009	20.09±0.43
	7.0	25-45%	178.0±0.23	1622.0±0.62	17.63±0.32	0.010±0.008	23.41±0.38
		45-65%	175.0±0.00	1649.7±0.52	21.10±0.40	0.012±0.001	28.02±0.47
	7.5	25-45%	175.0±0.35	1691.7±0.52	20.97±0.29	0.012±0.007	27.84±0.34
		45-65%	175.5±0.15	1885.7±0.24	24.13±0.50	0.013±0.012	32.04±0.58
	8.0	25-45%	175.0±0.00	1374.7±0.35	8.03±0.28	0.007±0.08	10.67±0.33
		45-65%	175.0±0.00	1488.3±0.48	10.81±0.28	0.009±0.006	13.62±0.27
60	6.5	25-45%	175.0±0.35	1330.3±2.60	6.52±0.42	0.005±0.003	8.66±0.12
		45-65%	175.5±0.15	1420.0±0.40	8.39±0.42	0.007±0.003	11.15±0.45
	7.0	25-45%	175.5±0.23	1504.7±0.33	13.30±0.17	0.009±0.003	17.66±0.24
		45-65%	175.0±0.35	1525.3±0.36	15.20±0.17	0.004±0.000	20.18±0.50
	7.5	25-45%	175.5±0.23	1557.3±0.26	15.73±0.23	0.010±0.000	20.88±0.24
		45-65%	175.0±0.35	1626.7±0.49	17.60±0.50	0.011±0.003	23.37±0.38
	8.0	25-45%	175.5±0.15	1254.3±0.48	6.34±0.24	0.005±0.007	7.42±0.09
		45-65%	175.0±0.00	1292.0±0.40	8.22±0.120	0.006±0.000	10.91±0.13

Replicates = 3

V : Volume
TP : Total protein content
TA : Total inhibitory activity
SA : Specific activity
Y : Yield

Table 6.14. Effects of incubation times, pH and ammonium sulphate saturation levels on TP, TA, SA and Y of cystatin extracted from bovine lung and pancreas mixture.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
15	6.5	25-45%	210.5±0.38	1957.3±0.63	64.33±0.32	0.033±0.007	21.59±0.19
		45-65%	212.5±0.65	2280.9±0.64	78.87±0.22	0.035±0.005	26.47±0.13
	7.0	25-45%	210.5±0.49	2315.0±0.33	95.40±0.27	0.041±0.006	32.02±0.16
		45-65%	212.3±0.54	2461.2±0.34	116.00±0.48	0.048±0.001	38.61±0.28
	7.5	25-45%	212.0±0.58	2649.5±0.29	118.50±0.24	0.045±0.005	39.77±0.14
		45-65%	212.5±0.31	2782.8±0.26	127.60±0.30	0.046±0.006	42.83±0.18
	8.0	25-45%	210.5±0.58	1783.7±3.49	46.30±0.16	0.026±0.0000	15.52±0.04
		45-65%	212.5±0.38	1995.2±0.19	62.80±0.20	0.032±0.004	21.10±0.12
30	6.5	25-45%	212.0±0.00	1832.1±1.72	82.55±0.45	0.044±0.000	27.71±0.13
		45-65%	212.5±0.31	1894.0±0.43	89.41±0.24	0.045±0.005	31.01±0.13
	7.0	25-45%	212.3±0.54	2021.5±0.20	99.98±0.78	0.049±0.005	33.56±0.12
		45-65%	210.5±0.49	2110.6±0.27	121.3±0.26	0.057±0.006	40.71±0.15
	7.5	25-45%	210.5±0.38	2328.1±0.30	135.90±0.29	0.058±0.006	45.61±0.17
		45-65%	212.5±0.65	2526.0±0.00	154.60±0.37	0.061±0.007	51.89±0.22
	8.0	25-45%	210.5±0.49	1621.7±0.40	58.45±0.28	0.036±0.007	19.62±0.16
		45-65%	212.3±0.54	1792.1±0.35	75.80±0.20	0.046±0.005	25.46±0.12

Replicates = 3

V : Volume

TP : Total protein content

TA : Total inhibitory activity

SA : Specific activity

Y : Yield

Table 6.14. Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
45	6.5	25-45%	210.5±0.38	1610.9±0.12	68.72±0.71	0.040±0.03	20.07±0.19
		45-65%	212.5±0.65	1717.4±0.46	77.06±1.24	0.042±0.03	25.86±0.72
	7.0	25-45%	210.5±0.49	1730.2±0.55	82.97±0.32	0.048±0.00	27.85±0.19
		45-65%	212.3±0.54	1792.3±0.38	92.79±0.78	0.052±0.03	31.14±0.2 0
	7.5	25-45%	212.3±0.54	1862.6±0.35	97.73±0.29	0.052±0.007	32.80±0.17
		45-65%	212.3±0.54	2036.5±0.30	114.2±0.40	0.059±0.005	38.33±0.11
	8.0	25-45%	210.5±0.49	1566.0±0.65	49.70±0.51	0.034±0.009	14.69±0.14
		45-65%	212.5±0.65	1584.1±0.34	68.39±2.01	0.046±0.008	20.63±0.55
60	6.5	25-45%	210.5±0.38	1429.0±2.98	48.81±2.70	0.030±0.005	16.38±0.75
		45-65%	212.5±0.65	1500.8±0.25	56.72±0.25	0.031±0.007	19.03±0.15
	7.0	25-45%	210.5±0.49	1595.4±0.51	64.21±0.44	0.035±0.011	21.55±0.26
		45-65%	212.3±0.54	1693.4±0.57	77.38±0.40	0.043±0.009	25.97±0.25
	7.5	25-45%	210.5±0.38	1628.5±0.19	76.22±0.73	0.042±0.004	25.58±0.11
		45-65%	212.5±0.65	1782.2±0.23	85.21±3.15	0.043±0.001	28.60±0.88
	8.0	25-45%	210.5±0.49	1335.3±0.35	37.36±0.54	0.020±0.003	12.54±0.15
		45-65%	212.3±0.54	1407.8±0.66	48.21±1.18	0.030±0.005	16.18±0.33

Replicates = 3

V : Volume

TP : Total protein content

TA : Total inhibitory activity

SA : Specific activity

Y : Yield

Table 6.15. Analysis of variance for total protein (TP) – ammonium sulphate extraction

Source	DF	SS	MS	F	P
Total	287	489370715			
Model					
ST	2	5789895	2894948	5034.5	0.001
IT	3	22664170	7554723	13138.0	0.001
PH	3	13647913	4549304	7911.5	0.001
SL	1	1250769	1250769	2175.1	0.001
ST*IT	6	1186343	197724	343.9	0.001
ST*PH	6	246917	41153	71.6	0.001
ST*SL	2	16656	8328	14.5	0.001
IT*PH	9	1755436	195048	339.2	0.001
IT*SL	3	262437	87479	152.1	0.001
PH*SL	3	75858	25286	44.0	0.001
ST*IT*PH	18	772091	42894	74.6	0.001
ST*IT*SL	6	128488	21415	37.2	0.001
IT*PH*SL	9	156341	17371	30.2	0.001
ST*IT*PH*SL	18	417402	23189	40.3	0.001
Error	198	113855	575		

ST : Sample type
IT : Incubation time (min)
PH : pH
SL : Saturation level (%)
DF : Degree of freedom
SS : Sum of square
MS : Mean of square
R^2 : 0.9977
CV : 2.1%

Table 6.16. Tukey grouping for total protein (TP) - ammonium sulphate extraction

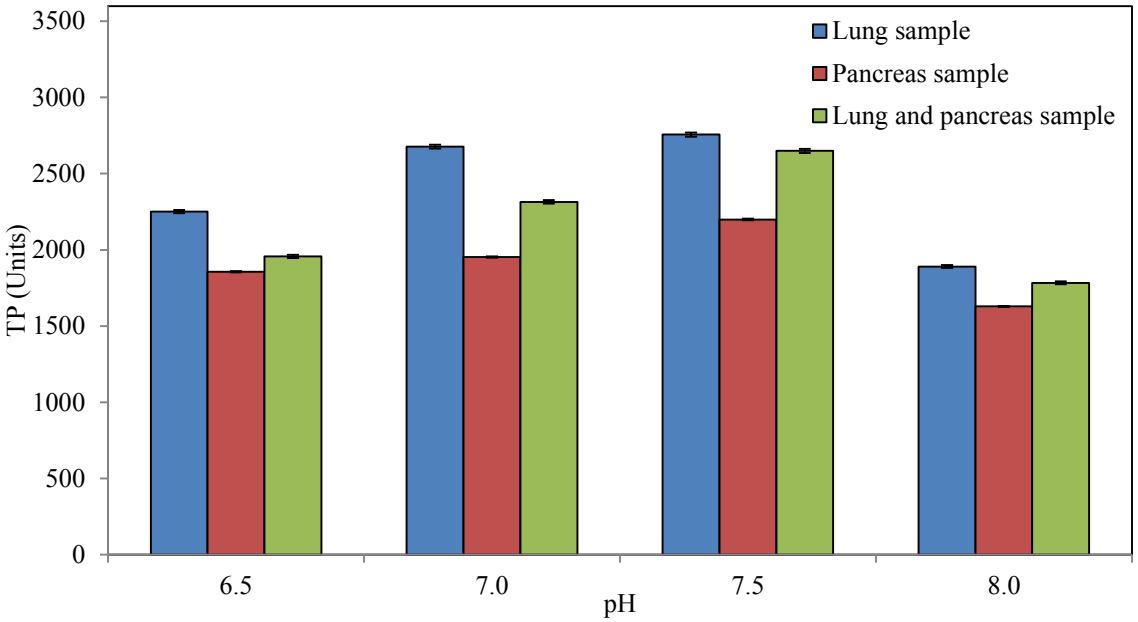
Factors	Level	N	Mean TP (%)	Tukey Grouping
Sample Type	Lung sample	96	2090	A
	Lung and pancreas sample	96	1888	B
	Pancreas sample	96	1744	C
Incubation Time (min)	15	72	2277	A
	30	72	2063	B
	45	72	1735	C
	60	72	1555	D
pH	7.5	72	2216	A
	7.0	72	1987	B
	6.5	72	1794	C
	8.0	72	1633	D
Saturation Level (%)	45-65	144	1973	A
	25-45	144	1841	B

Means that share the same letter are not significantly different from one another at the 0.05 level

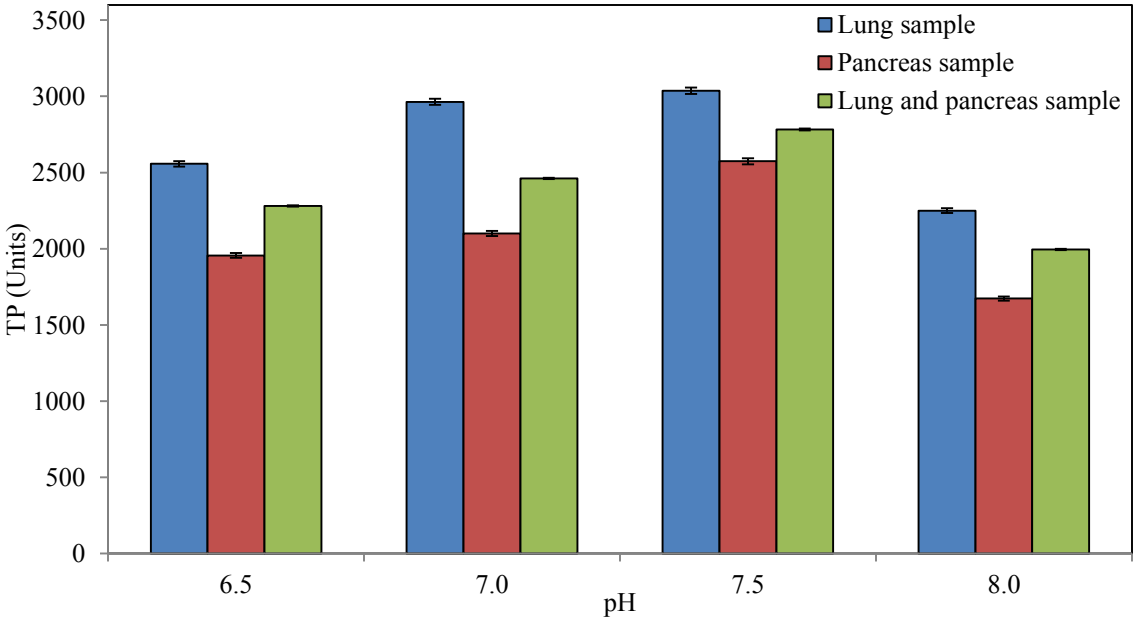
total protein (TP) mean of 2216 mg was obtained at the pH 7.5. The ammonium sulphate saturation levels were also significantly different from each other at the 0.05 level. The highest average total protein (TP) mean of 1973 mg was obtained with the 45-65% ammonium sulphate saturation.

6.2.1.1. Effect of Sample Type on Total Protein (TP): The effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on total protein (TP) of cystatin extracted at different incubation times (15, 30, 45 and 60 min), pH levels (6.5, 7.0, 7.5, and 8.0) and ammonium sulphate saturation levels (25-45% and 45-65%) are shown in Figures 6.1- 6.4. With the 25-45% ammonium sulphate saturation, pH 6.5 and 15 min incubation, the total protein (TP) of the cystatin extracted from the bovine lung was 2251 mg, which was higher than those extracted from the pancreas and mixture of lung and pancreas samples All three samples (bovine lung, pancreas and mixture of lung and pancreas) exhibited similar trends with the 45-65% ammonium sulphate saturation, all pH levels and incubation times. The bovine lung sample showed higher total protein (TP) of cystatin than the pancreas and mixture of lung and pancreas samples under all conditions.

6.2.1.2. Effect of pH on Total Protein (TP): The effect of pH (6.5, 7.0, 7.5, and 8.0) on total protein (TP) of cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different incubation times (15, 30, 45 and 60 min) and ammonium sulphate saturation levels (25-45% 45-65%) are shown in Figures 6.5 - 6.7. The SD for the graphs are less (<2). With the 25-45% ammonium sulphate saturation, increasing the pH from 6.5 to 7.5 increased the TP of cystatin extracted from bovine lung sample from 2251 to 2756.7 mg (22.47%), from 2069.7 to 2549.2 mg (23.17%), from 1790 to 2055.3 mg (14.82%) and from 1558.3 to 1717.2 mg (10.2%) for the incubation times 15, 30, 45 and 60 min, respectively. A further increase in the pH from 7.5 to 8.0 decreased the TP of cystatin from 2756.7 to 1890.3 mg (31.42%), from 2549.2 to 1715.1 mg (32.72%), from 2055.3 to 1609.7 mg (21.68%) and from 1717.2 to 1418.4 mg (17.4%) for the incubation times of 15, 30, 45 and 60 min, respectively. Similar trends were observed with the bovine

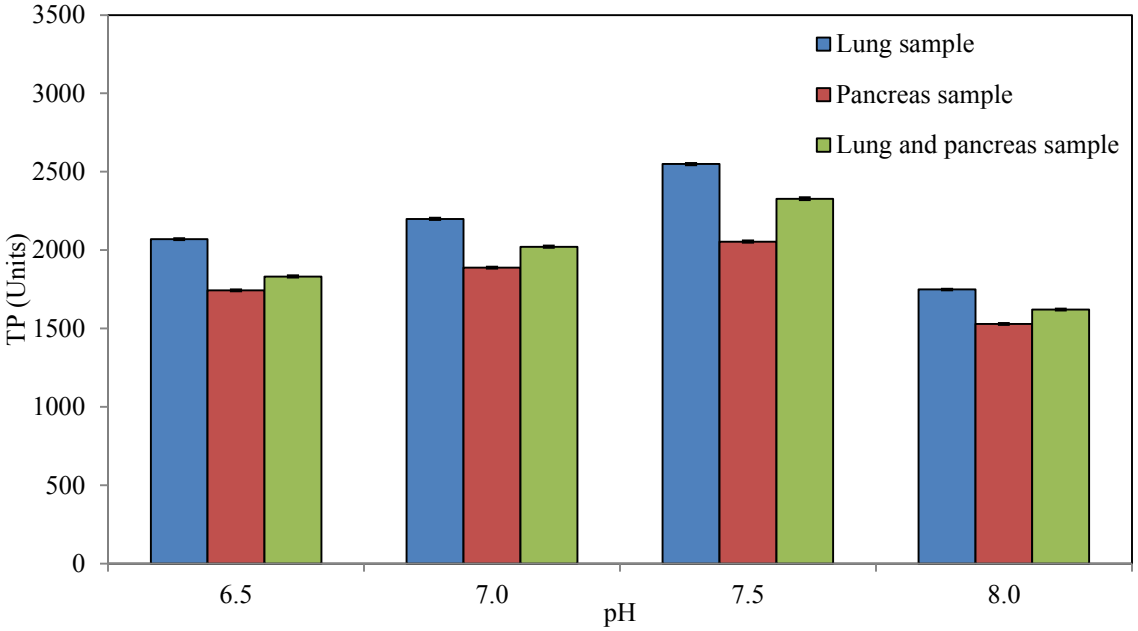


(a) 25-45 % ammonium sulphate saturation

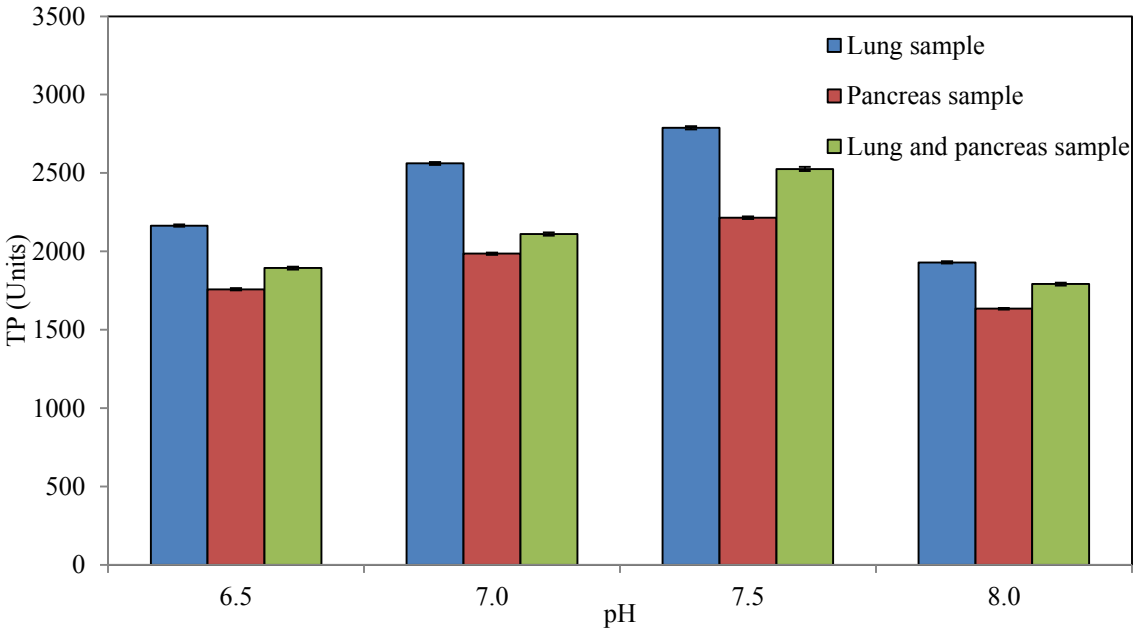


(b) 45-65 % ammonium sulphate saturation

Figure 6.1. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on the total protein (TP) of the cystatin extracted at 15 min incubation and different levels pH and ammonium sulphate saturation

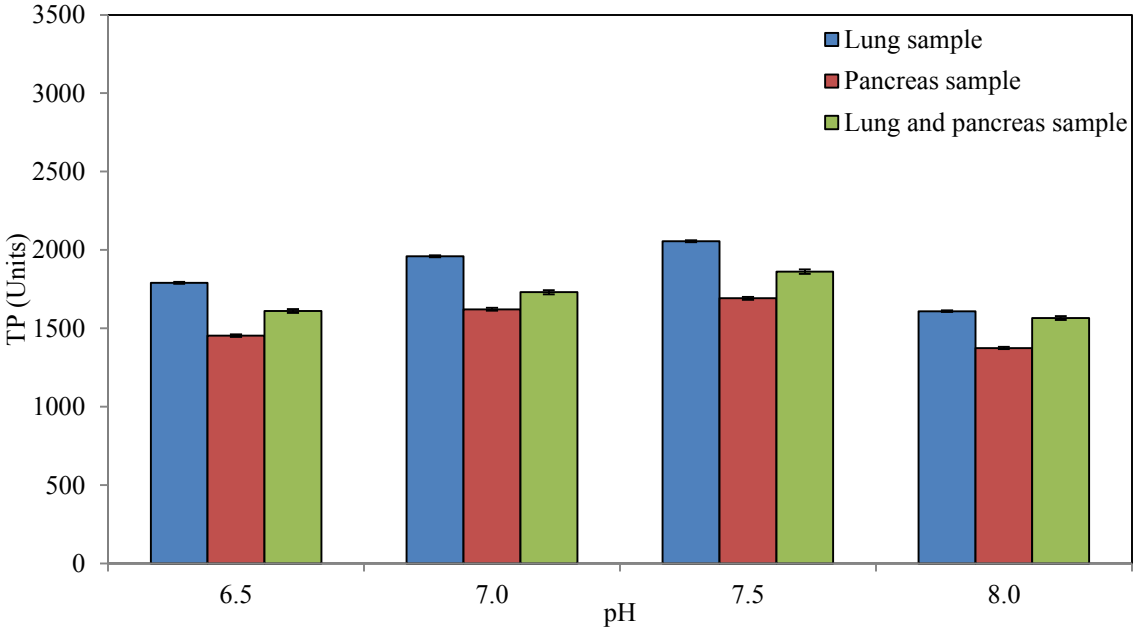


(a) 25-45 % ammonium sulphate saturation

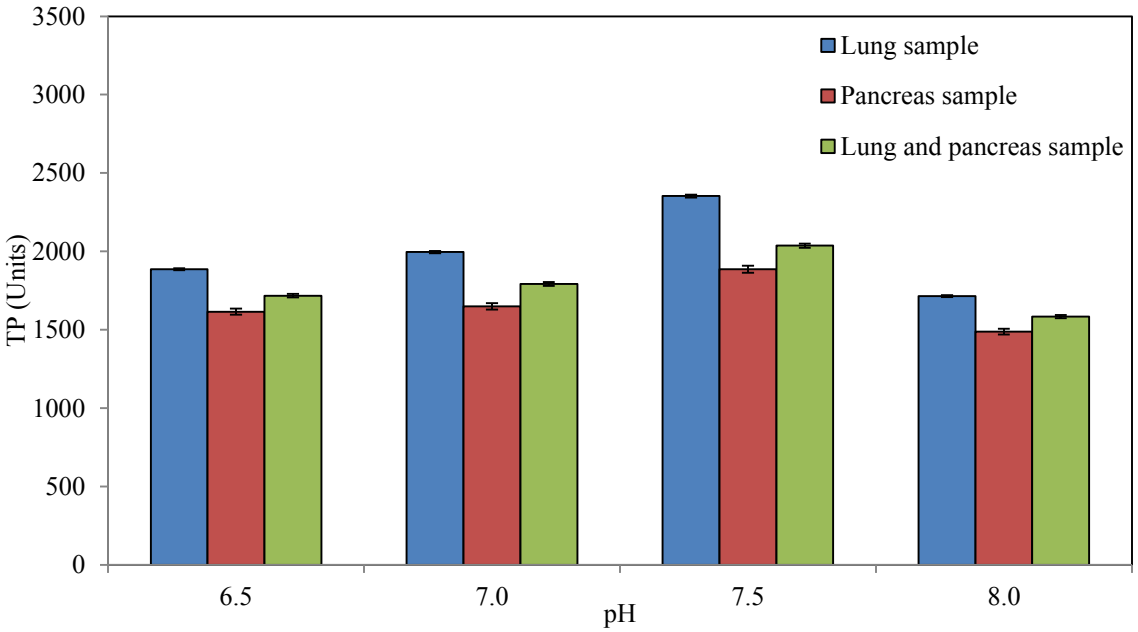


(b) 45-65 % ammonium sulphate saturation

Figure 6.2. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on the total protein (TP) of the cystatin extracted at 30 min incubation and different levels pH and ammonium sulphate saturation

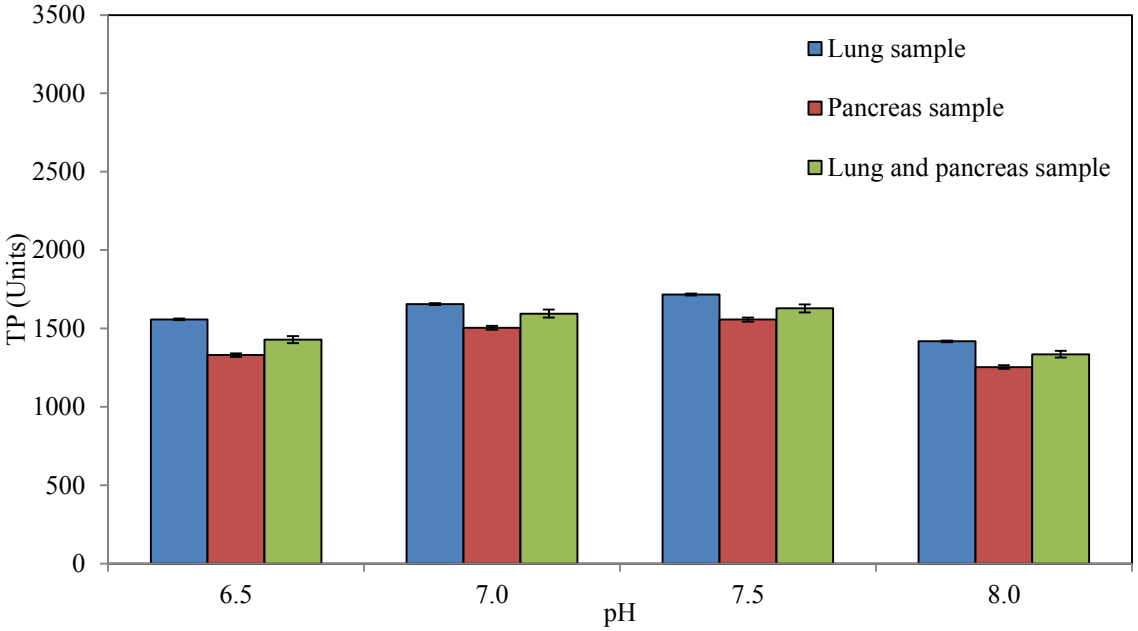


(a) 25-45 % ammonium sulphate saturation

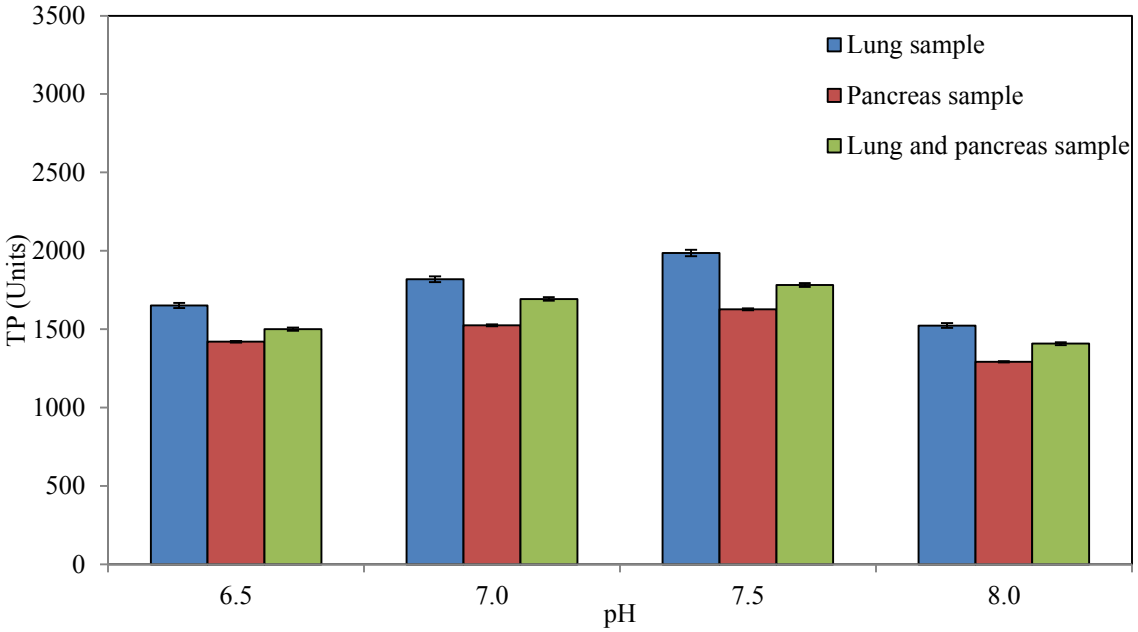


(b) 45-65 % ammonium sulphate saturation

Figure 6.3. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on the total protein (TP) of the cystatin extracted at 45 min incubation and different levels pH and ammonium sulphate saturation

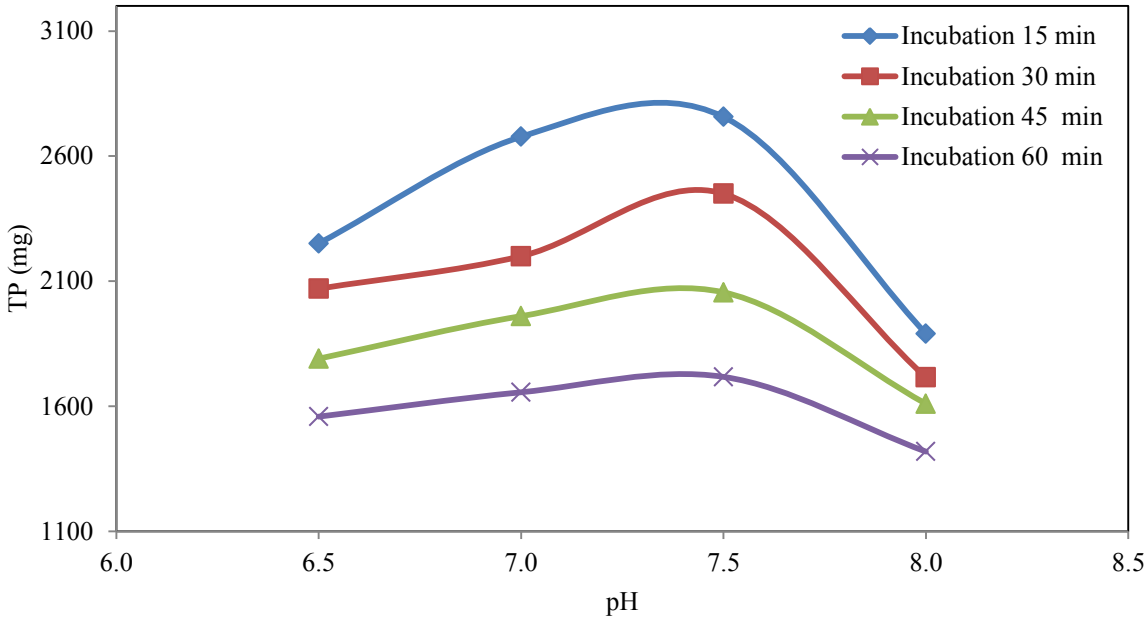


(a) 25-45 % ammonium sulphate saturation

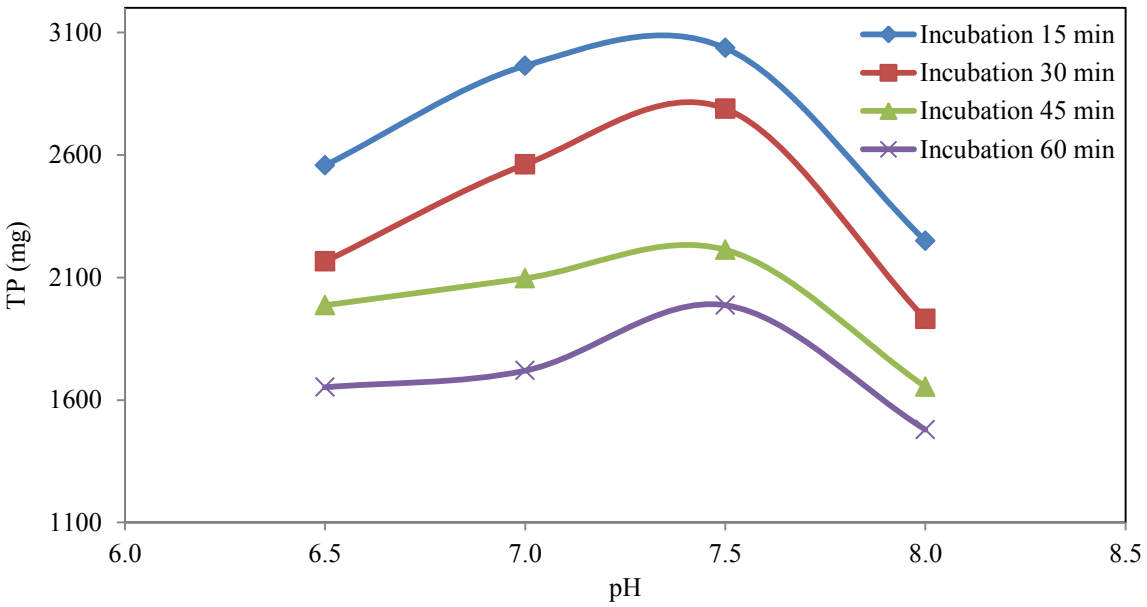


(b) 25-45 % ammonium sulphate saturation

Figure 6.4. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on the total protein (TP) of the cystatin extracted at 60 min incubation and different levels pH and ammonium sulphate saturation

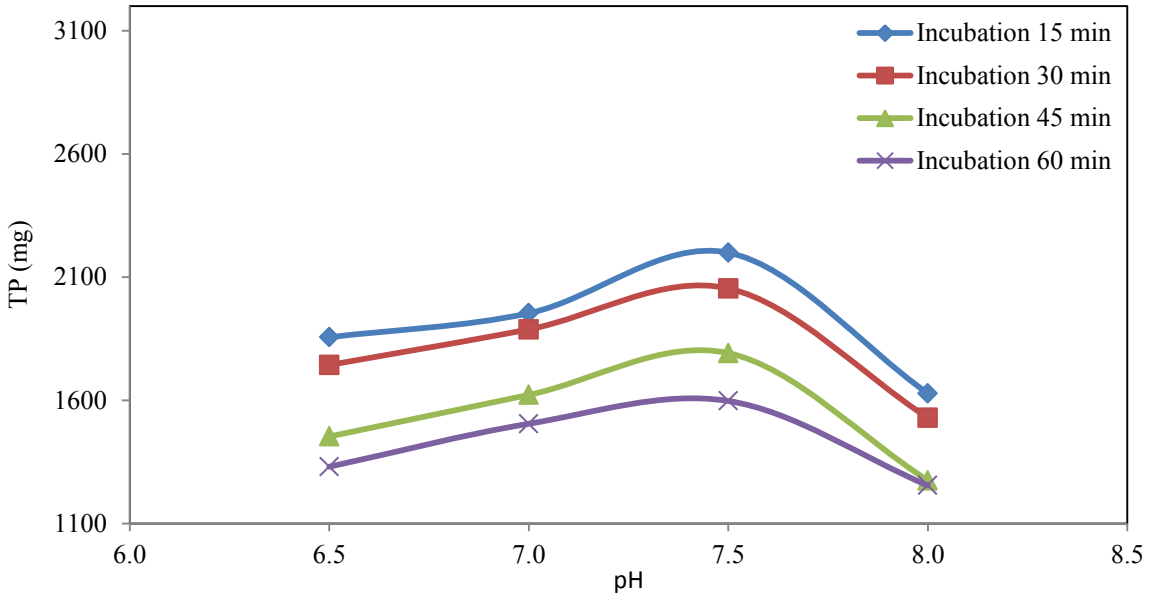


(a) 25-45% ammonium sulphate saturation

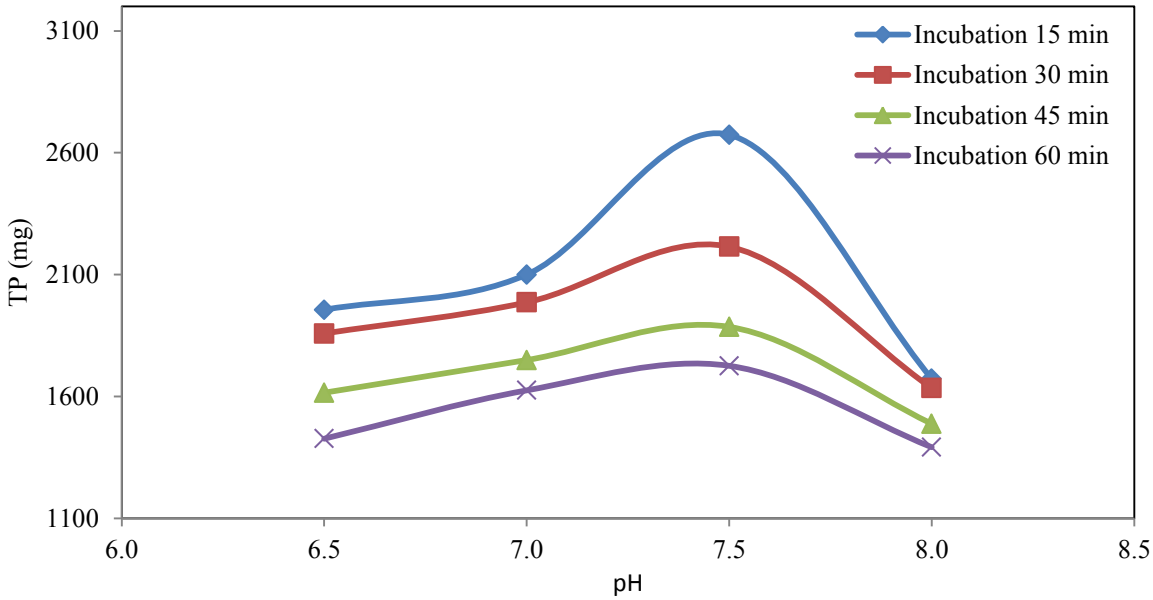


(b) 45-65% ammonium sulphate saturation

Figure 6.5. Effect of pH on the total protein (TP) of the cystatin extracted from bovine lung at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3)

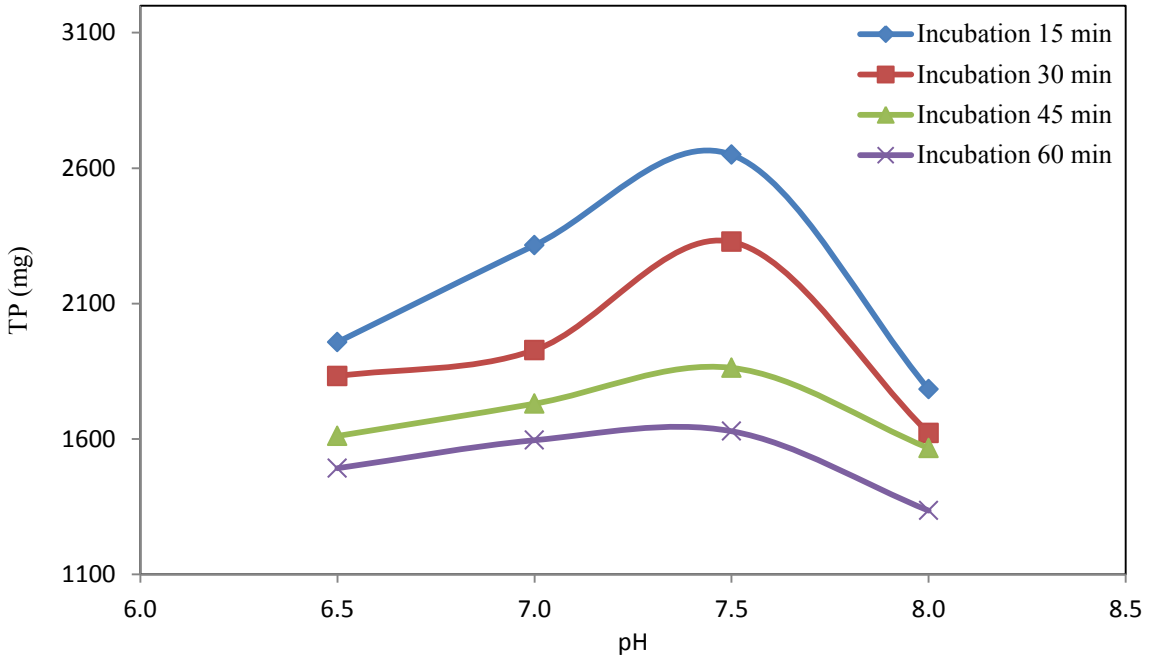


(a) 25-45% ammonium sulphate saturation

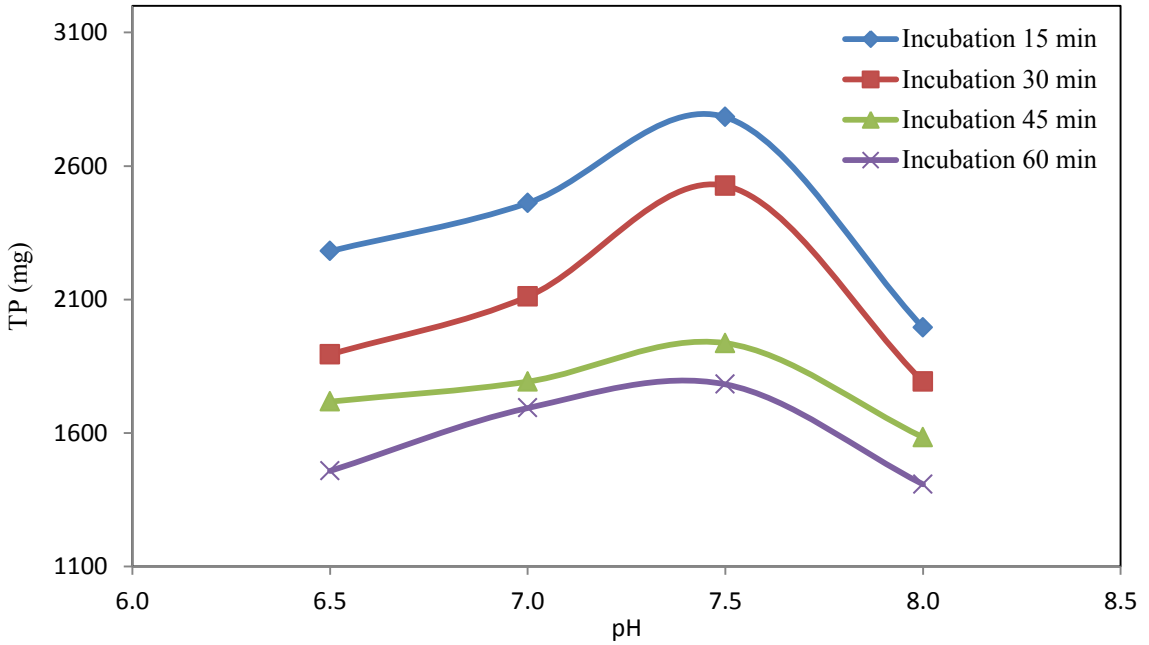


(b) 45-65% ammonium sulphate saturation

Figure 6.6. Effect of pH on the total protein (TP) of the cystatin extracted from bovine pancreas at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3).



(a) 25-45% ammonium sulphate saturation



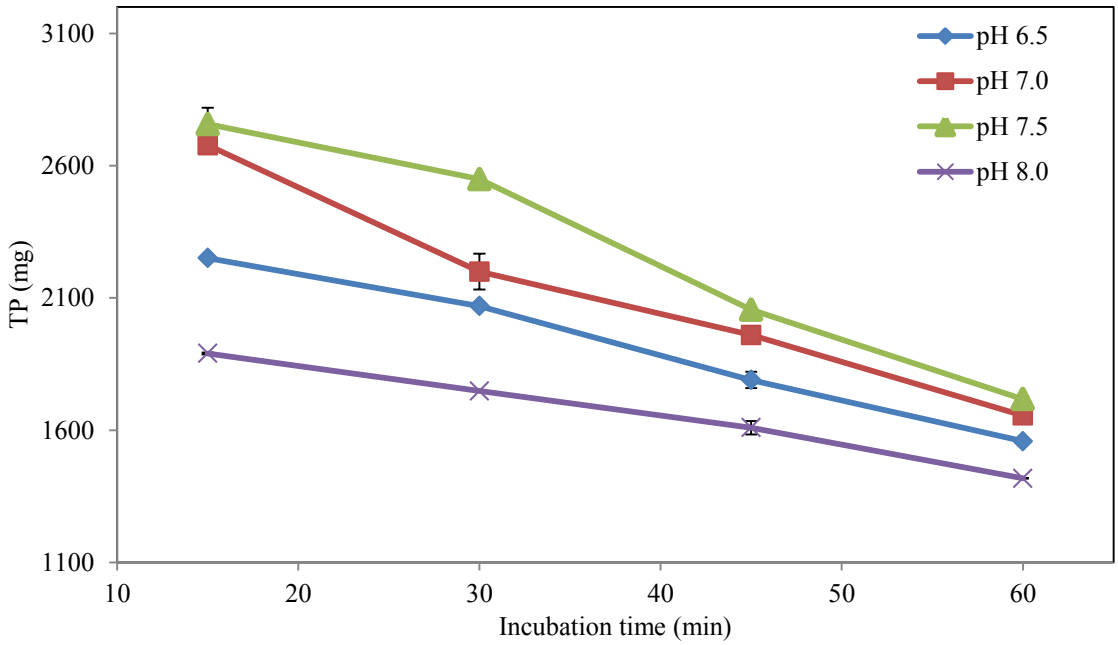
(b) 45-65% ammonium sulphate saturation

Figure 6.7. Effect of pH on the total protein (TP) of the cystatin extracted from bovine lung and pancreas mixture at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3).

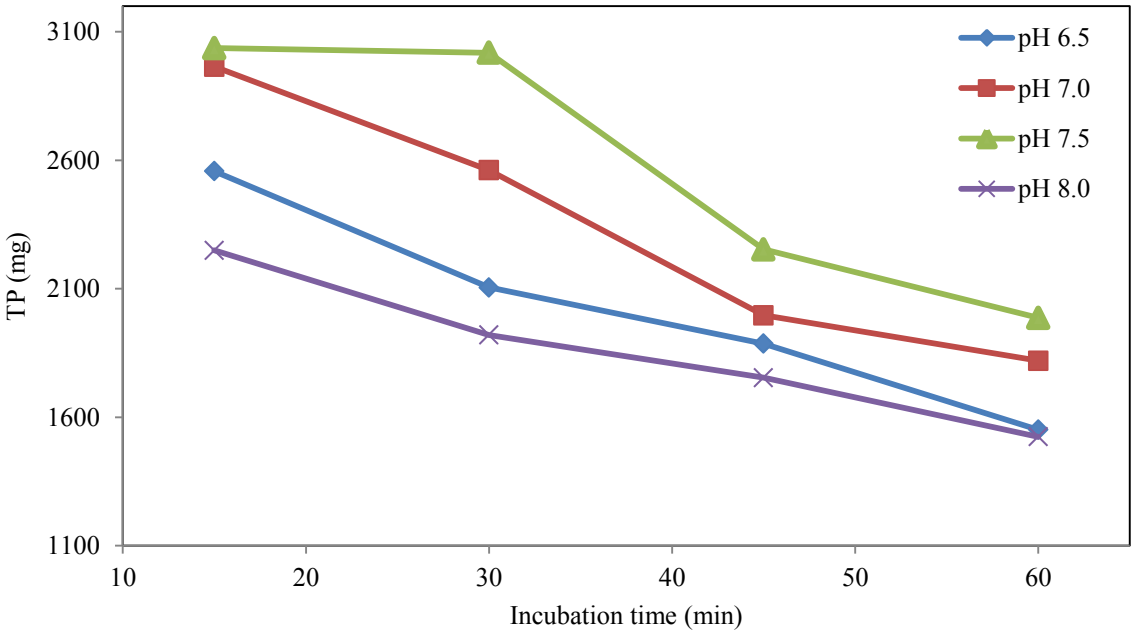
pancreas and the mixture of lungs and pancreas samples. All the three samples (bovine lung, pancreas and mixture of lungs and pancreas samples) also exhibited similar trends ammonium sulphate at all incubation times. The pH 7.5 produced the highest TP of cystatin extracted from the samples under all incubation times.

6.2.1.3. Effect of incubation on Total Protein (TP): The effect of incubation time (15, 30, 45 and 60 min) on the total protein (TP) of the cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different pH levels (6.5, 7.0, 7.5, and 8.0) and ammonium sulphate saturation levels (25-45% and 45-65%) are shown in Figures 6.8- 6.10. With the 25-45% ammonium sulphate saturation, increasing the incubation time from 15 to 60 min decreased the TP of the cystatin extracted from the bovine lung samples from 2251 to 1558 mg (30.77%), 2677.3 to 1655.7 mg (38.15%), 2756.7 to 1717.2 mg (37.70%) and 1890.3 to 1418.4 mg (24.96%) at the pH levels of 6.5, 7.0, 7.5 and 8.0, respectively. Similar trends were observed for the TP of the cystatin extracted from the bovine pancreas and the mixture of lung and pancreas samples. All the three samples (bovine lung, pancreas and mixture of lungs and pancreas samples) also exhibited similar trends with the 45-65% ammonium sulphate saturation. The highest TP of cystatin was observed at the incubation time of 15 min for all the samples at all pH levels.

6.2.1.4. Effect of ammonium sulphate saturation on Total Protein (TP): The effect of ammonium sulphate saturation (25-45% and 45-65%) on the total protein (TP) of the cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different pH levels (6.5, 7.0, 7.5, and 8.0) and incubation times (15, 30, 45 and 60min) are shown in in Figures 6.11- 6.13. With the 15 min incubation time, increasing the ammonium sulphate saturation from 25-45% to 45-65% increased the TP of the cystatin extracted from bovine lung samples from 2251 to 2557.7 mg (13.62%), from 2677.3 to 2964 mg (10.70%), from 2756.7 to 3036.3 mg (10.14%) and from 1890.3 to 2249.7 mg (19.01%) at pH levels 6.5, 7.0, 7.5 and 8.0, respectively. Similar trends were observed for the TP of the cystatin extracted from the three samples (bovine lung sample, pancreas sample and mixture of lungs and pancreas sample) at other incubation times

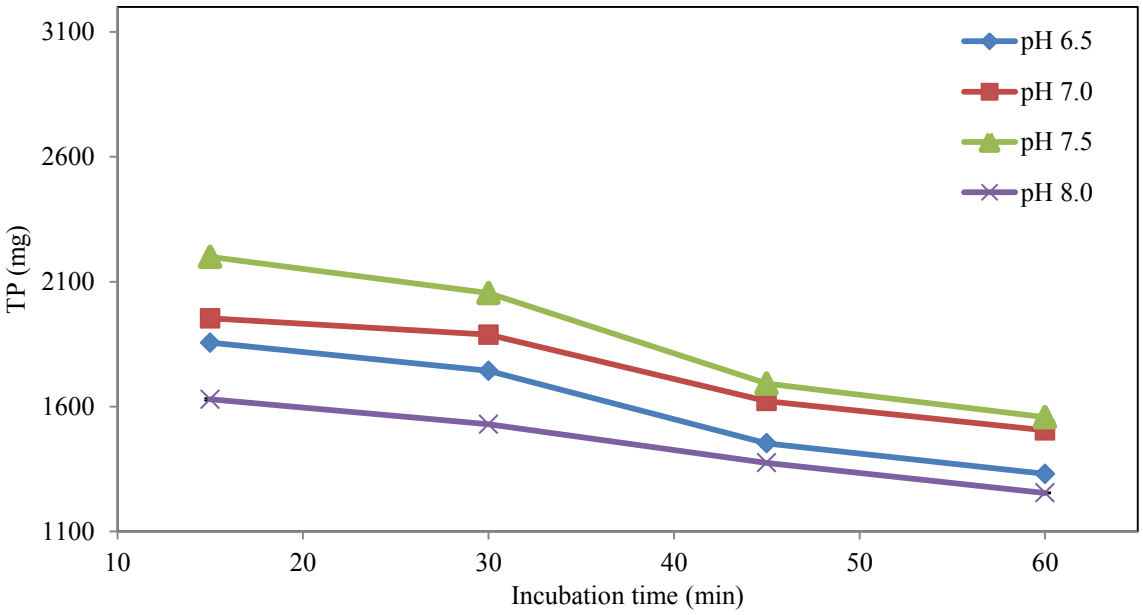


(a) 25-45% ammonium sulphate saturation

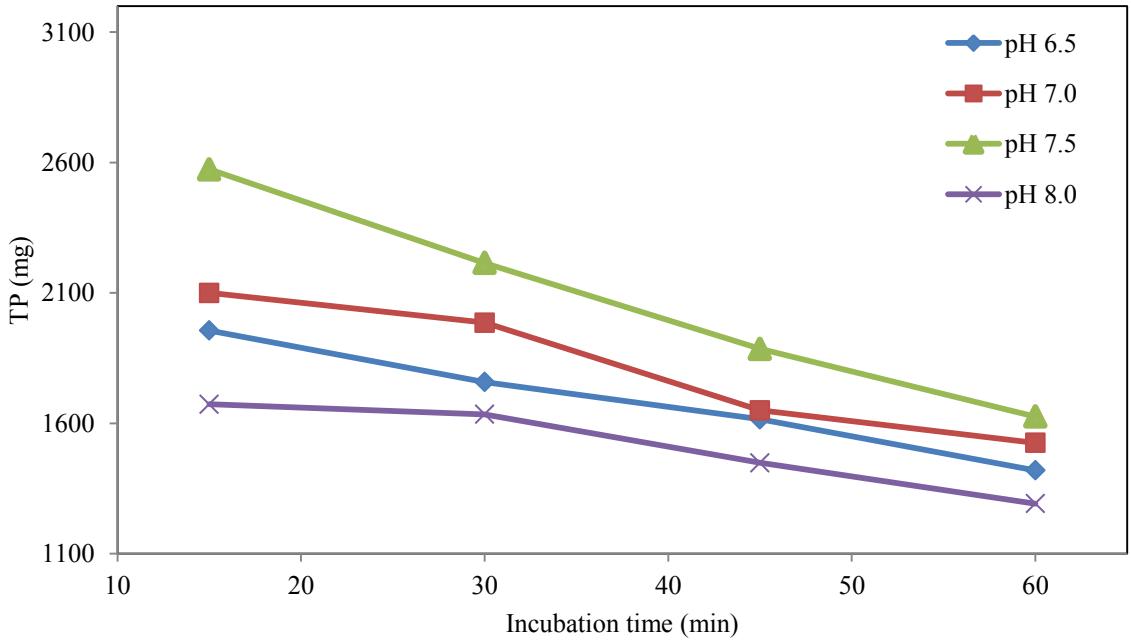


(b) 45-65% ammonium sulphate saturation

Figure 6.8. Effect of incubation time on the total protein (TP) of the cystatin extracted from the bovine lung at different pH and ammonium sulphate saturation levels (mean ± std, n=3).

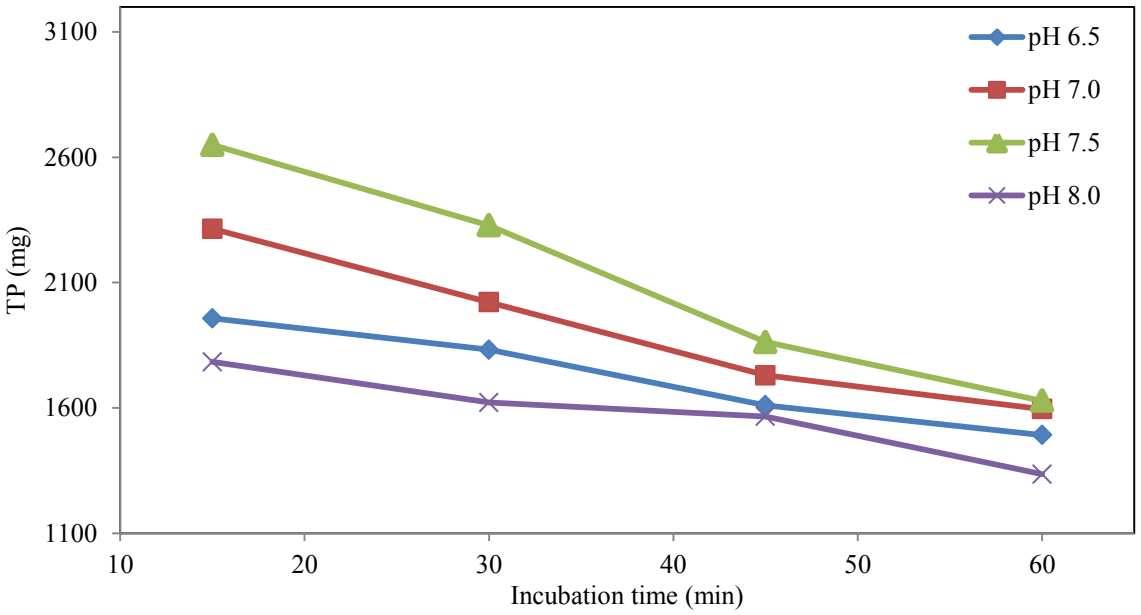


(a) 25-45% ammonium sulphate saturation

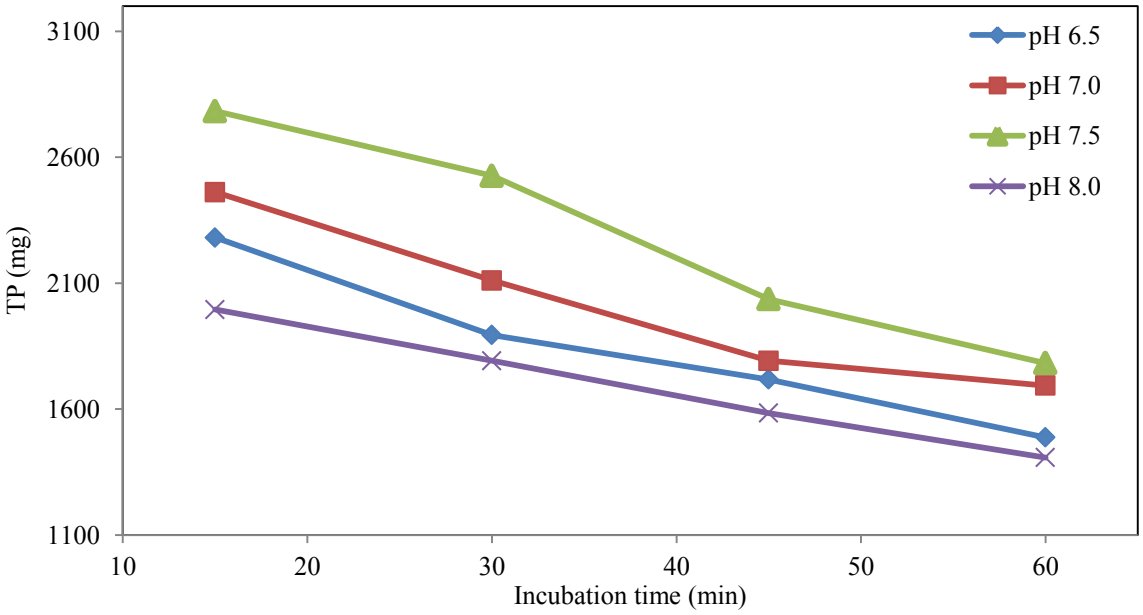


(b) 45-65% ammonium sulphate saturation

Figure 6.9. Effect of incubation time on the total protein (TP) of the cystatin extracted from the bovine pancreas at different pH and ammonium sulphate saturation levels (mean ± std, n=3).

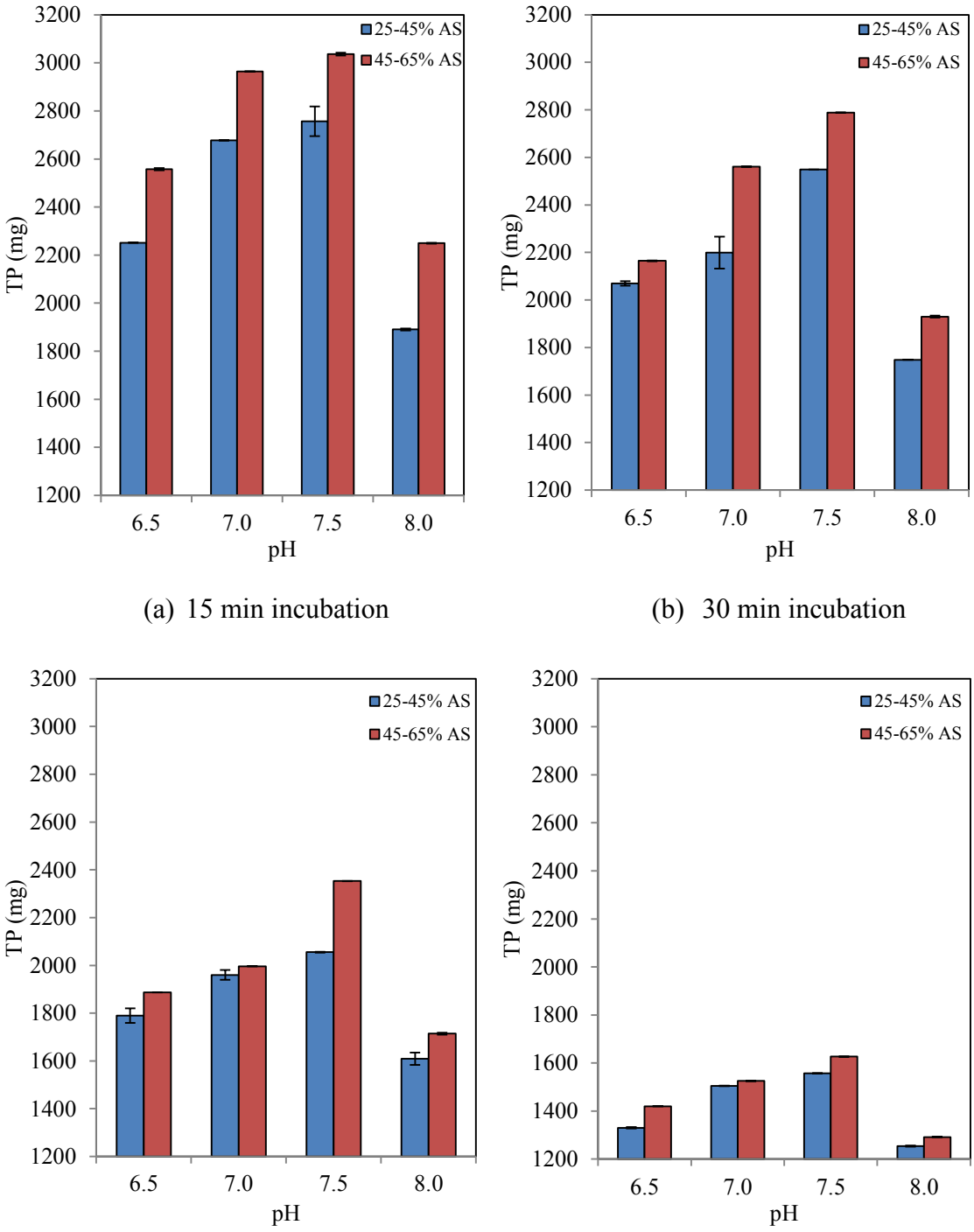


(a) 25-45% ammonium sulphate saturation



(b) 45-65% ammonium sulphate saturation

Figure 6.10. Effect of incubation time on the total protein (TP) of the cystatin extracted from the bovine lung and pancreas mixture at different pH and ammonium sulphate saturation levels (mean ± std, n=3)



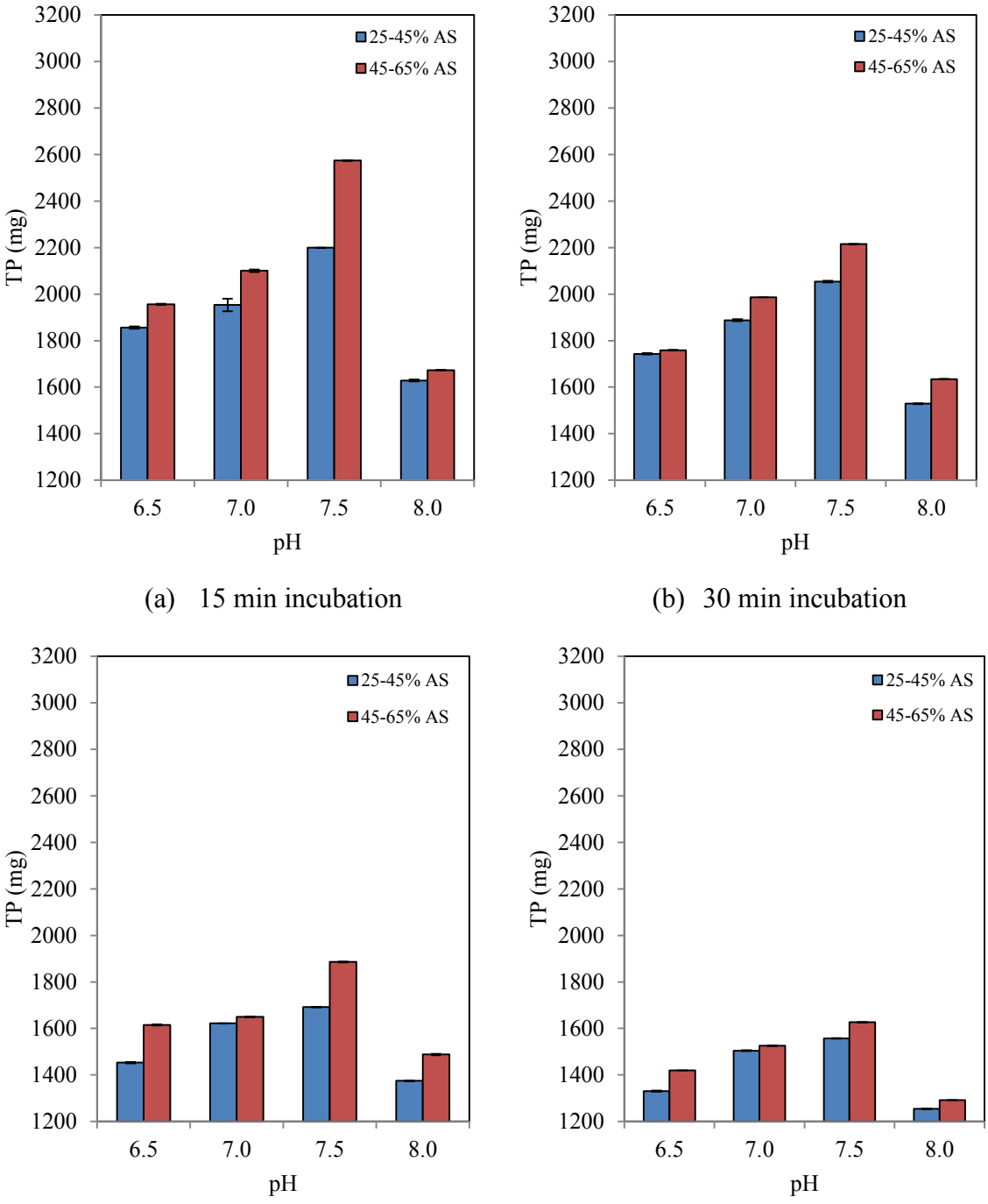
(a) 15 min incubation

(b) 30 min incubation

(c) 45 min incubation

(d) 60 min incubation

Figure 6.11. Effect of ammonium sulphate (AS) saturation on the total protein (TP) of the cystatin extracted from the bovine lung at different pH levels and incubation times (mean ± std, n=3)



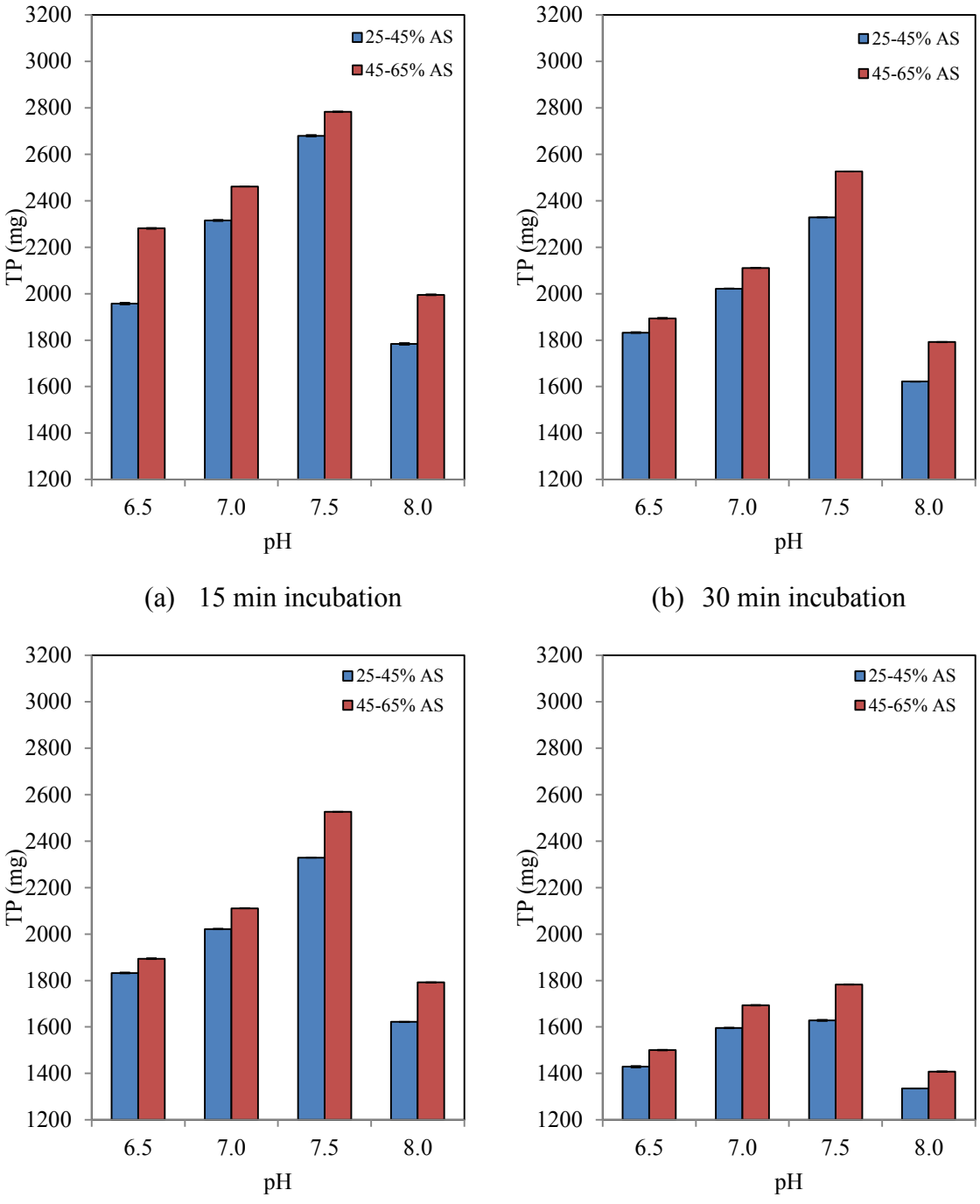
(a) 15 min incubation

(b) 30 min incubation

(c) 45 min incubation

(d) 60 min incubation

Figure 6.12. Effect of ammonium sulphate (AS) saturation on the total protein (TP) of the cystatin extracted from the bovine pancreas at different pH levels and incubation times (mean ± std, n=3).



(a) 15 min incubation

(b) 30 min incubation

(c) 45 min incubation

(d) 60 min incubation

Figure 6.13. Effect of ammonium sulphate (AS) saturation on the total protein (TP) of the cystatin extracted from the bovine lung and pancreas mixture at different pH levels and incubation times (mean ± std, n=3).

(15, 30, 45 and 60 min). The highest TP of the cystatin was observed at the 45-65% ammonium sulphate saturation for all the three samples under all conditions.

6.2.2. Total Activity (TA)

The effects of sample types (bovine lung, pancreas and mixture of lung and pancreas), pH (6.5, 7.0, 7.5, and 8.0), incubation time (15, 30, 45 and 60 min) and ammonium sulphate saturation level (25-45% and 45-65%) on the total activity (TA) of the cystatin are shown in Tables 6.12 - 6.14. The total activity (TA) of the cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas varied from 203 to 164 Units, from 14.40 to 8.22 Units and 64.33 to 48.21 Units, respectively.

Analysis of variance was performed on the total protein data using Minitab (Version 16.2.3, Minitab Inc, State College, Pennsylvania, USA). The results are shown in Table 6.17. The effects of sample type, pH level, incubation times and ammonium sulphate saturation were significant at the 0.001 level. The two, three and four way interactions between the parameters were also significant at the 0.001 level.

The Tukey grouping was also performed on the total activity (TA) data as shown in Table 6.18. The bovine lung, pancreas and mixture of lung and pancreas samples were significantly different from each other at the 0.05 level. The highest average total activity (TA) of cystatin of 272.56 Units was obtained from the bovine lung sample. All the incubation times were significantly different from each other at the 0.05 level. The highest average total activity (TA) of cystatin of 151.89 Units was obtained with the 30 min incubation time. All pH levels were significantly different from each other at the 0.05 level. The highest average total activity (TA) of cystatin of 169.56 Units was obtained at a pH of 7.5. The ammonium sulphate saturation levels were also significantly different from each other at the 0.05 level. The highest average total activity (TA) mean of 134.58 Units was obtained with the 45-65% ammonium sulphate saturation.

6.2.2.1. Effect of Sample Type on Total Activity (TA): The effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on the total activity (TA) of the cystatin extracted at different incubation times (15, 30, 45 and 60 min) and ammonium

Table 6.17. Analysis of variance for total activity (TA) – ammonium sulphate extraction

Source	DF	SS	MS	F	P
Total	287	4266707			
Model					
ST	2	3382455	1691227	44735.1	0.001
IT	3	126837	42279	1118.3	0.001
PH	3	301031	100344	2654.2	0.001
SL	1	30510	30510	807.0	0.001
ST*IT	6	95096	15849	419.2	0.001
ST*PH	6	217926	36321	960.7	0.001
ST*SL	2	24061	12031	318.2	0.001
IT*PH	9	31391	3488	92.3	0.001
IT*SL	3	5101	1700	45.0	0.001
PH*SL	3	3563	1188	31.4	0.001
ST*IT*PH	18	27565	1531	40.5	0.001
ST*IT*SL	6	8015	1336	35.3	0.001
IT*PH*SL	9	2558	284	7.5	0.001
ST*IT*PH*SL	18	3112	173	4.6	0.001
Error	198	7485	38		

ST : Sample type
IT : Incubation time (min)
PH : pH
SL : Saturation level (%)
DF : Degree of freedom
SS : Sum of square
MS : Mean of square
R^2 : 0.998
CV : 9.8%

Table 6.18. Tukey grouping for total activity (TA)- ammonium sulphate extraction

Factors	Level	N	Mean TA (Units)	Tukey Grouping
Sample Type	Lung sample	96	272.56	A
	Lung and pancreas sample	96	83.74	B
	Pancreas sample	96	16.56	C
Incubation Time (min)	30	72	151.89	A
	15	72	131.11	B
	45	72	120.50	C
	60	72	93.65	D
pH	7.5	72	169.56	A
	7.0	72	137.77	B
	6.5	72	105.46	C
	8.0	72	84.35	D
Saturation Level (%)	45-65	144	134.58	A
	25-45	144	113.99	B

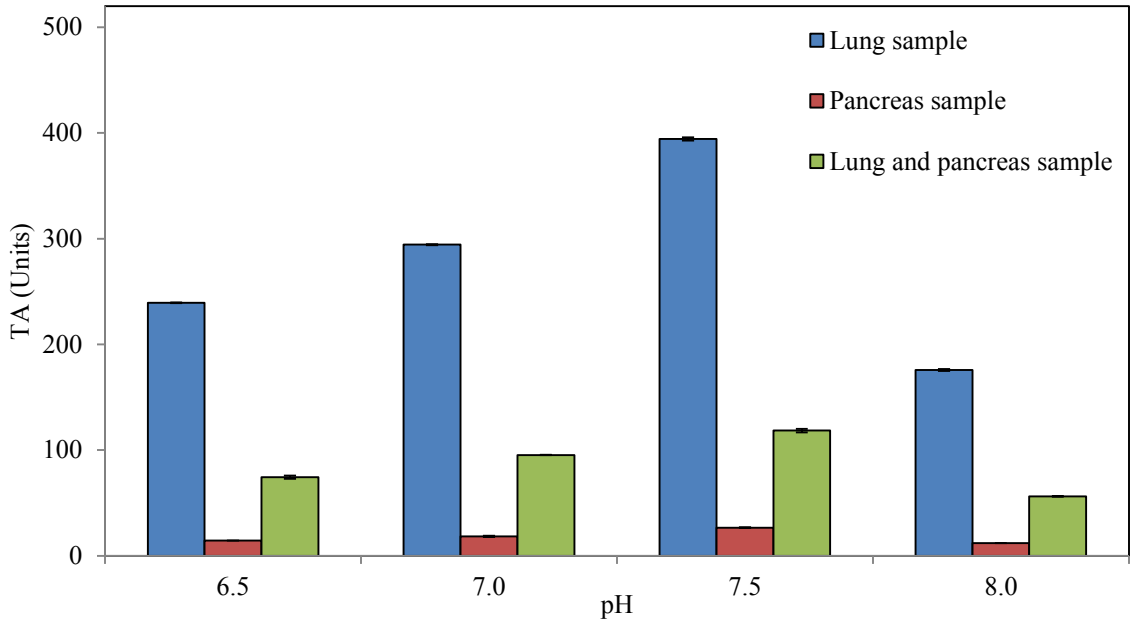
Means that share the same letter are not significantly different from one another at the 0.05 level.

sulphate saturation levels (25-45% and 45-65%) are shown in Figures 6.14 - 6.17. With the 25-45 % saturation, pH 6.5 and 15 min incubation, the total activity (TA) of the cystatin extracted from the bovine lung was 203 Units, which was higher than the pancreas and mixture of lung and pancreas samples All three samples (bovine lung, pancreas and mixture of lung and pancreas) exhibited similar trends with the 45-65% ammonium sulphate saturation and all pH levels and incubation times. The Bovine lung sample showed higher total activity (TA) of cystatin than the pancreas and mixture of lung and pancreas samples under all conditions.

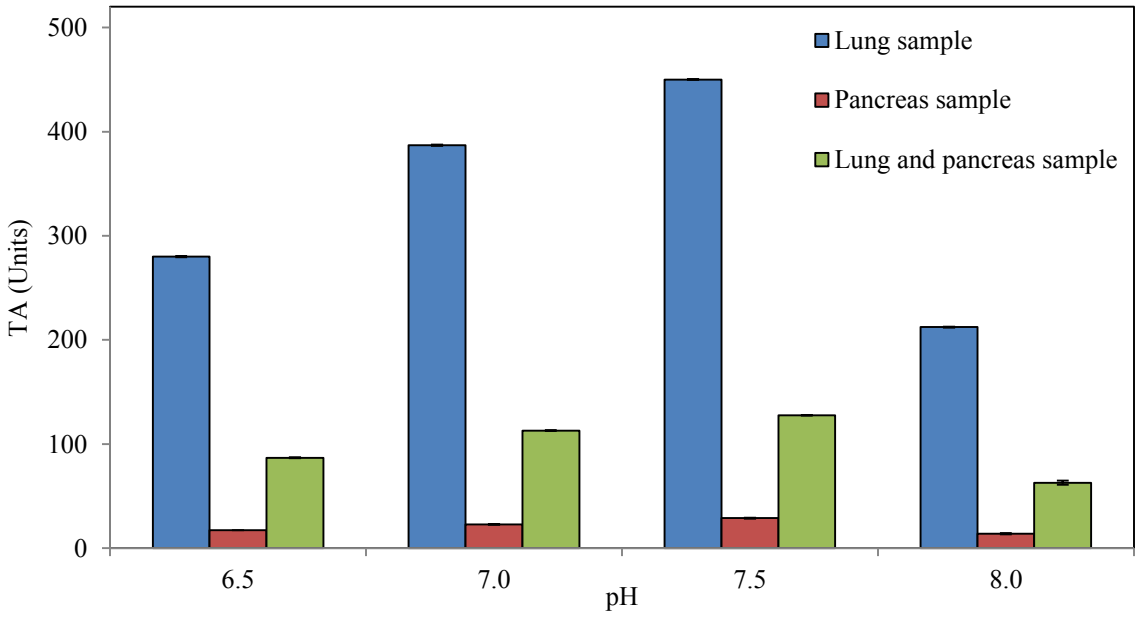
6.2.2.2. Effect of pH on Total Activity (TA): The effect of pH (6.5, 7.0, 7.5, and 8.0) on the total activity (TA) of cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different incubation times (15, 30, 45 and 60 min) and saturation levels (25-45% 45-65%) were shown in Figures 6.18 - 6.20. The SD for the graphs are too small (<2).With the 25-45% ammonium sulphate saturation, increasing the pH from 6.5 to 7.5 increased the TA of the cystatin extracted from the bovine lung sample from 239.3 to 394.4 Units (68.8%), from 276.4 to 439.4 Units (58.9%), from 203 to 350.7 Units (72.75%) and from 154.2 to 269 mg (74.4%) for the incubation times of 15, 30, 45 and 60 min, respectively. A further increase in the pH (from 7.5 to 8.0) decreased the TA of cystatin from 394.4 to 175.8 Units (55.4%), from 439.4 to 188.7 Units (57%), 350.7 to 156.3 (55.4%) and from 269 to 144 mg (46.4%) for the incubation times of 15, 30, 45 and 60 min respectively. Similar trends were observed with the bovine pancreas and the mixture of lungs and pancreas samples. All the three samples (bovine lung, pancreas and mixture of lungs and pancreas samples) also exhibited similar trends with the 45-65% ammonium sulphate saturation at all incubation times. The pH 7.5 produced the highest TA of cystatin extracted from all samples under all incubation times.

6.2.2.3. Effect of incubation on Total Activity (TA): The effect of incubation time (15, 30, 45 and 60 min) on the total activity (TA) of cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different pH (6.5, 7.0, 7.5, and 8.0)

and ammonium sulphate saturation levels (25-45% and 45-65%) are shown in Figures 6.21 - 6.23. With the 25-45% ammonium sulphate saturation, increasing the incubation

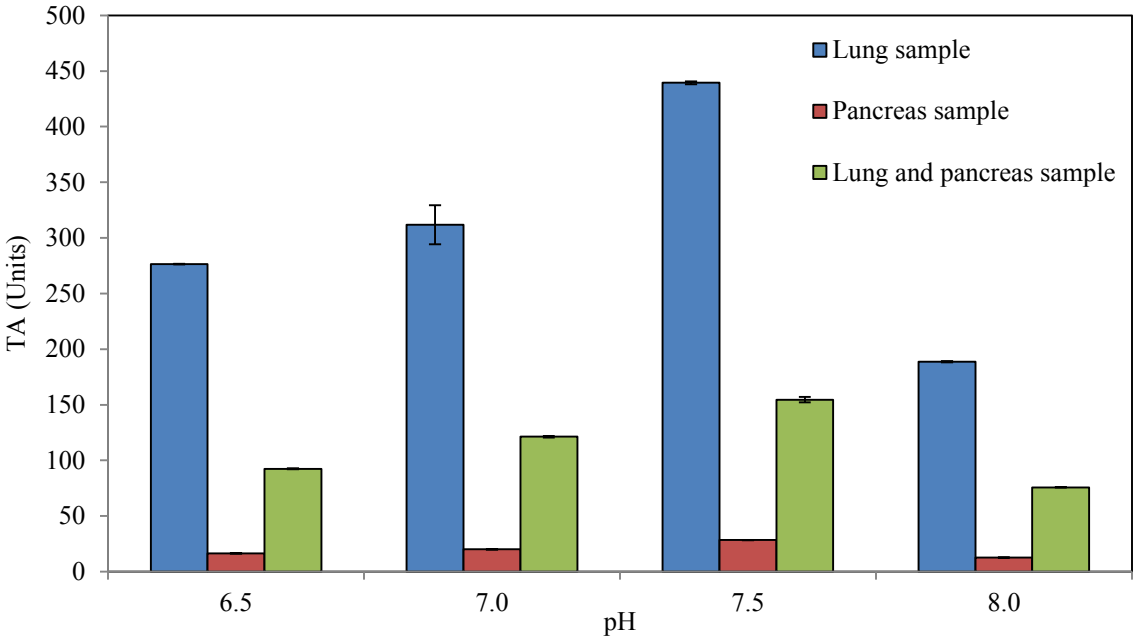


(a) 25-45% ammonium sulphate saturation

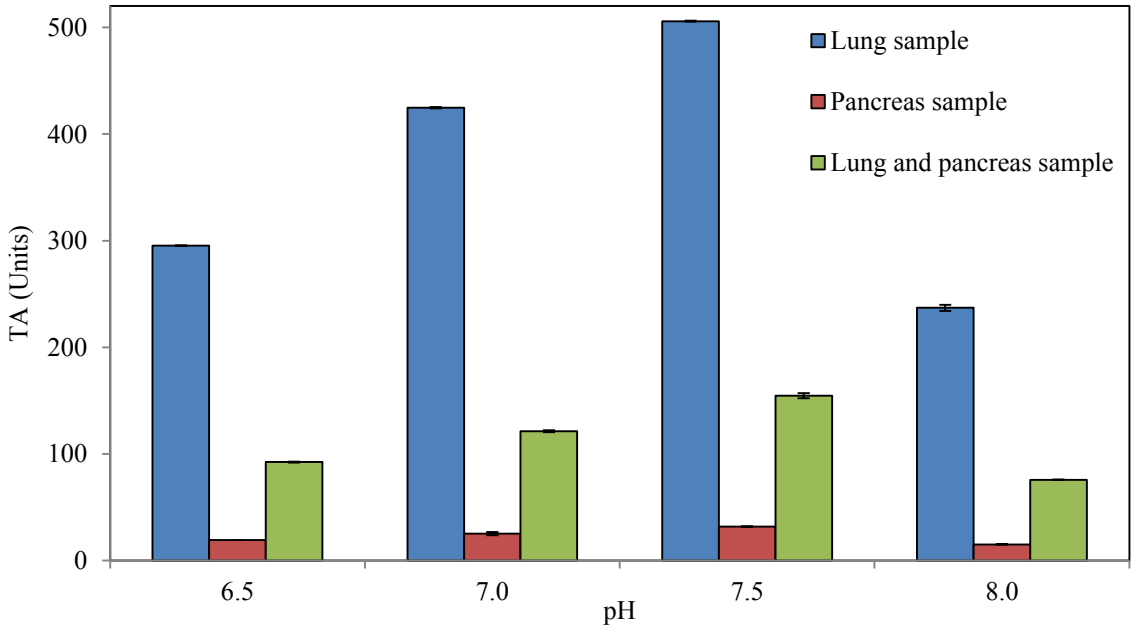


(b) 45-65% ammonium sulphate saturation

Figure 6.14. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on the total activity (TA) of the cystatin extracted at 15 min incubation and different levels of pH and ammonium sulphate saturation

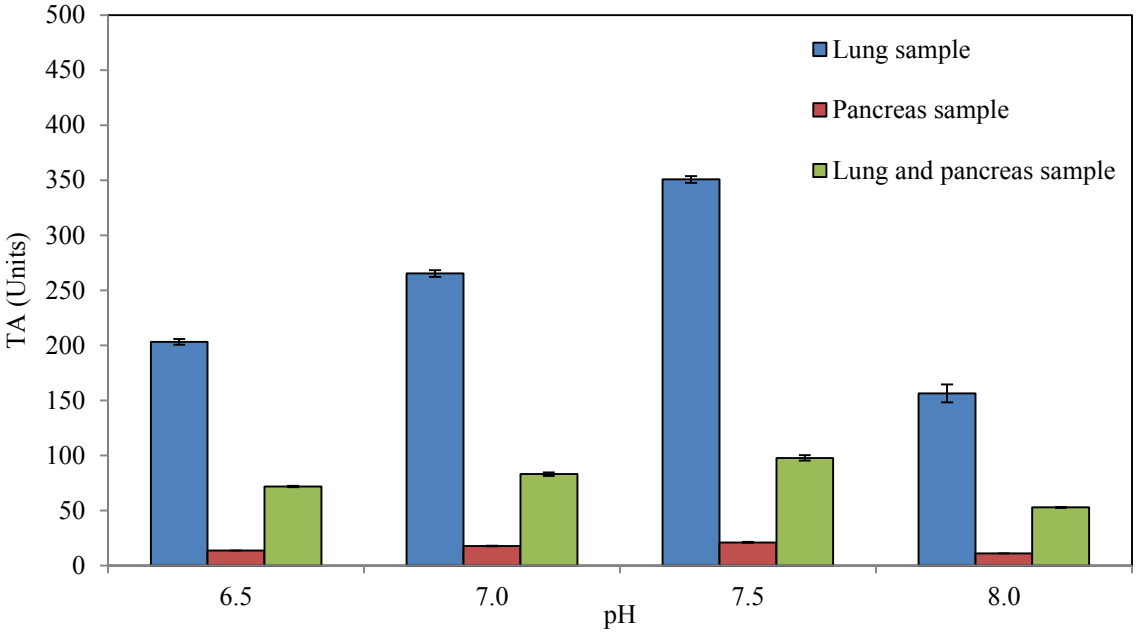


(a) 25-45% ammonium sulphate saturation

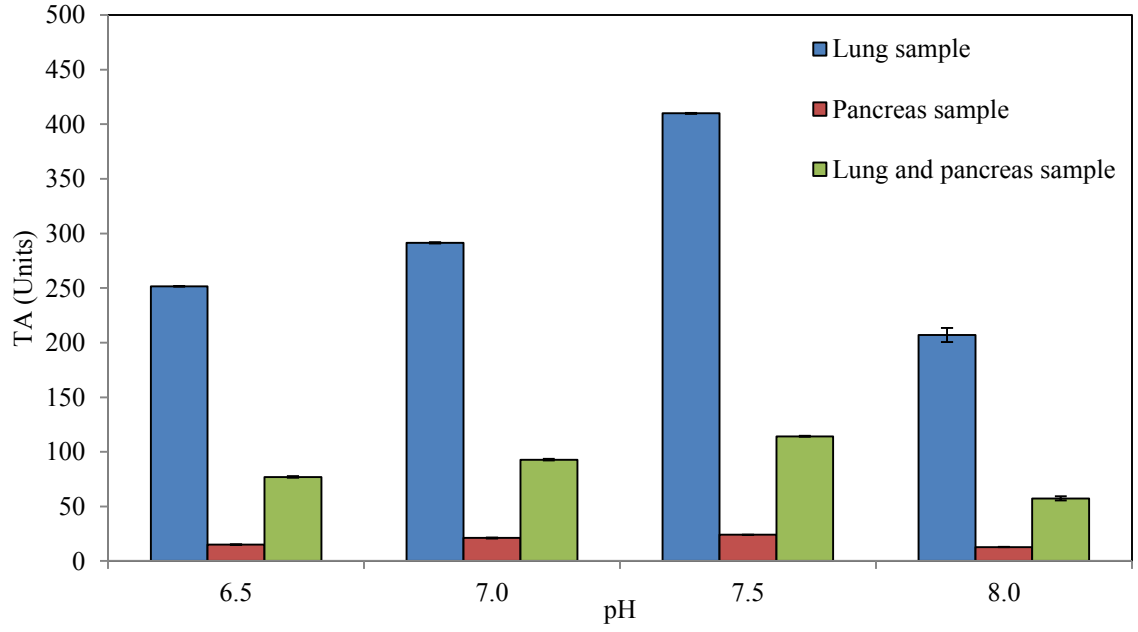


(b) 45-65% ammonium sulphate saturation

Figure 6.15. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on total activity (TA) of the cystatin extracted at 30 min incubation and different levels of pH and ammonium sulphate saturation

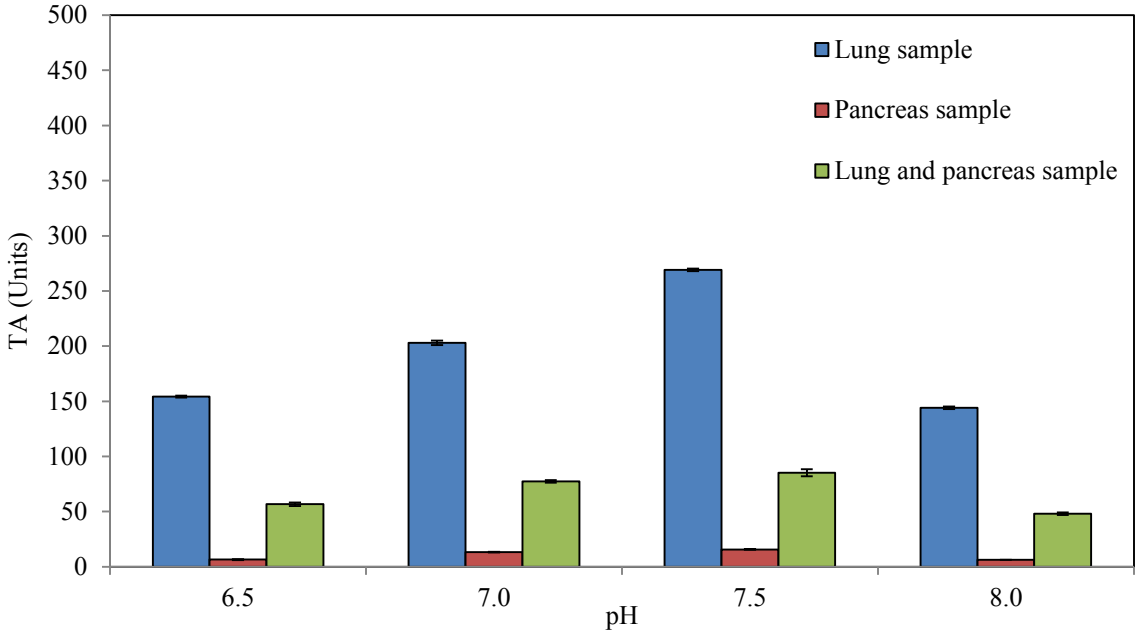


(a) 25-45% ammonium sulphate saturation

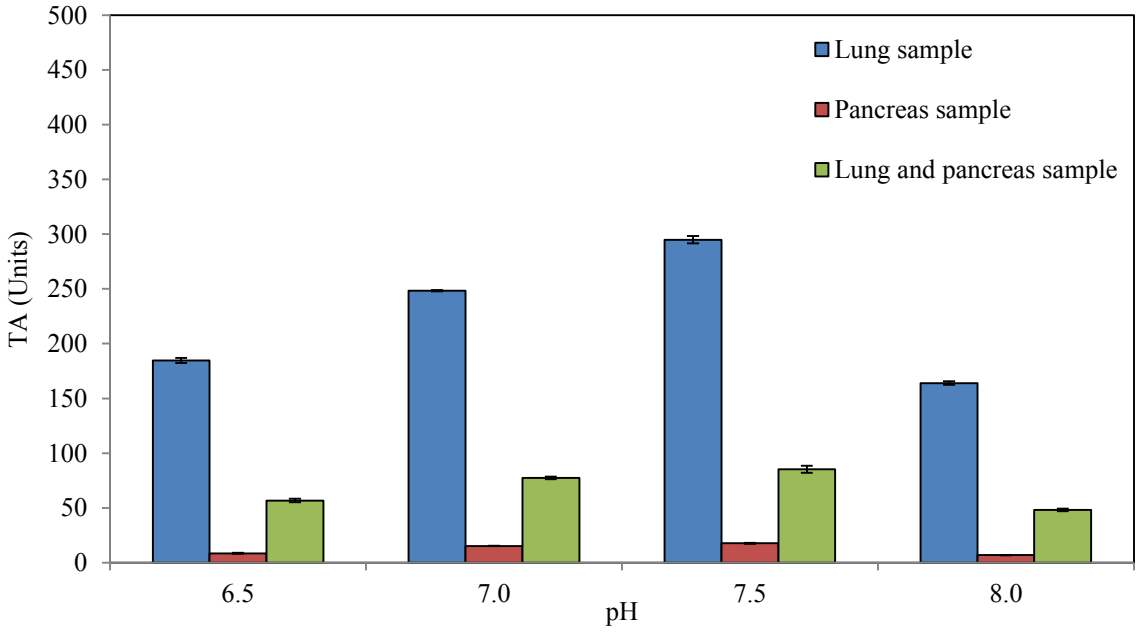


(b) 45-65% ammonium sulphate saturation

Figure 6.16. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on total activity (TA) of the cystatin extracted at 45 min incubation and different levels of pH and ammonium sulphate saturation

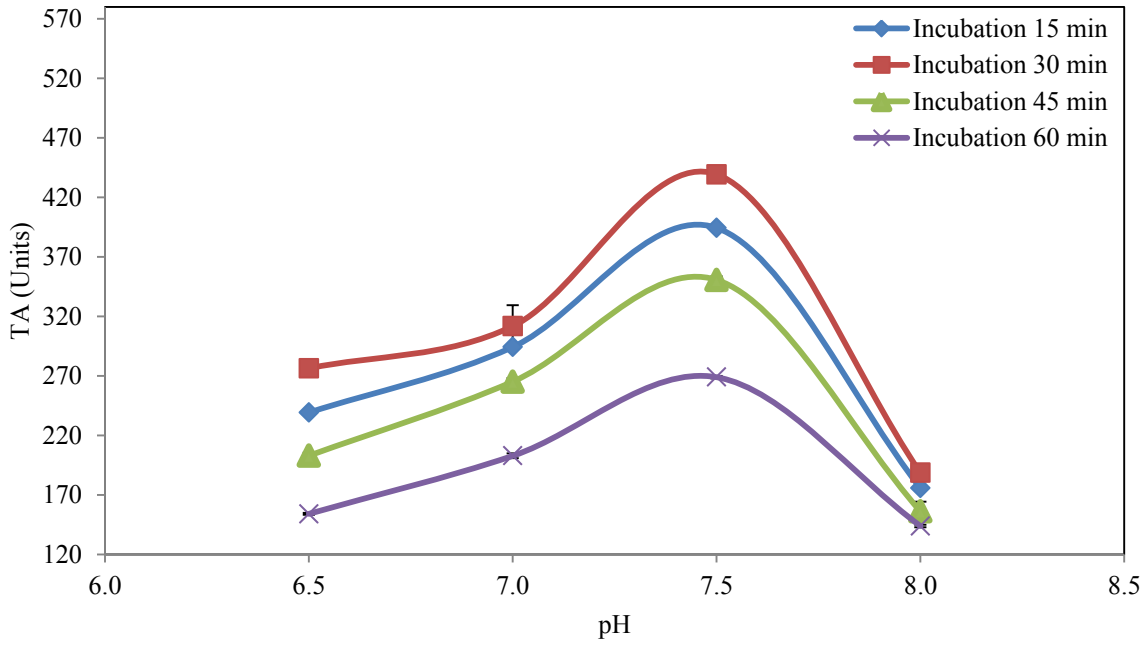


(a) 25-45% ammonium sulphate saturation

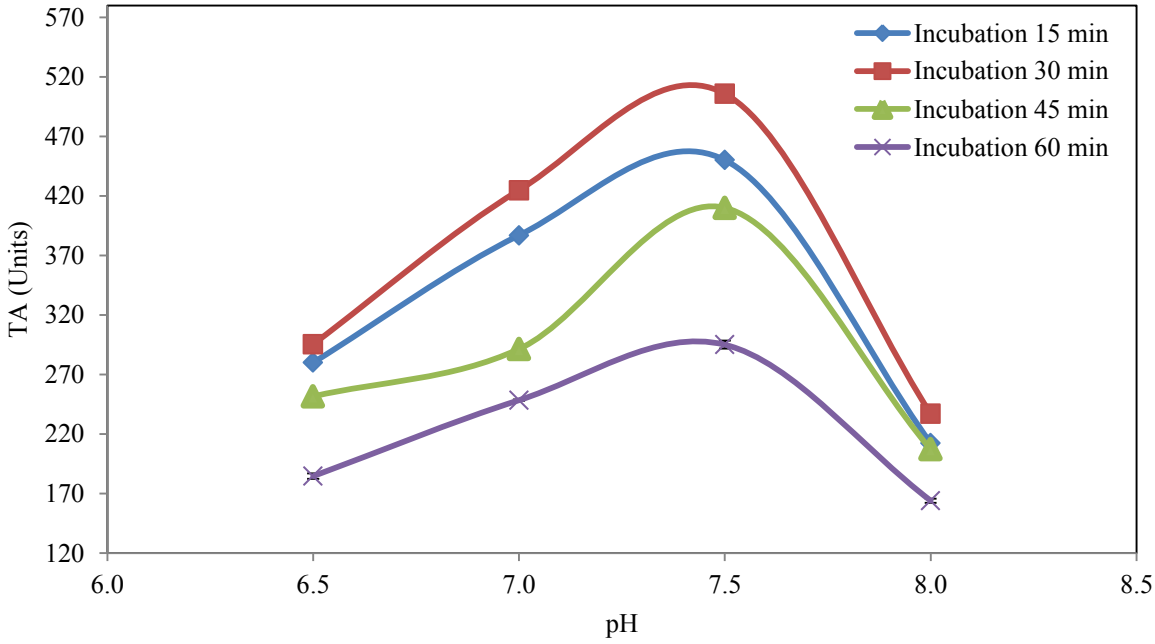


(b) 45-65% ammonium sulphate saturation

Figure 6.17. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on total activity (TA) of the cystatin extracted at 60 min incubation and different levels of pH and ammonium sulphate saturation

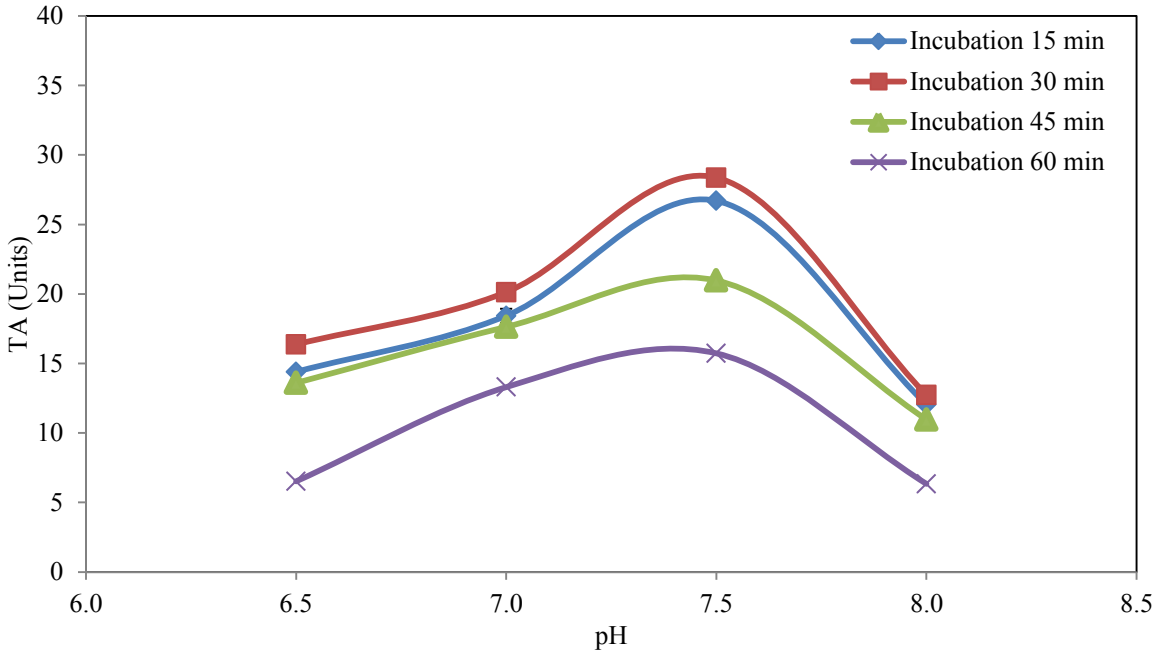


(a) 25-45% ammonium sulphate saturation

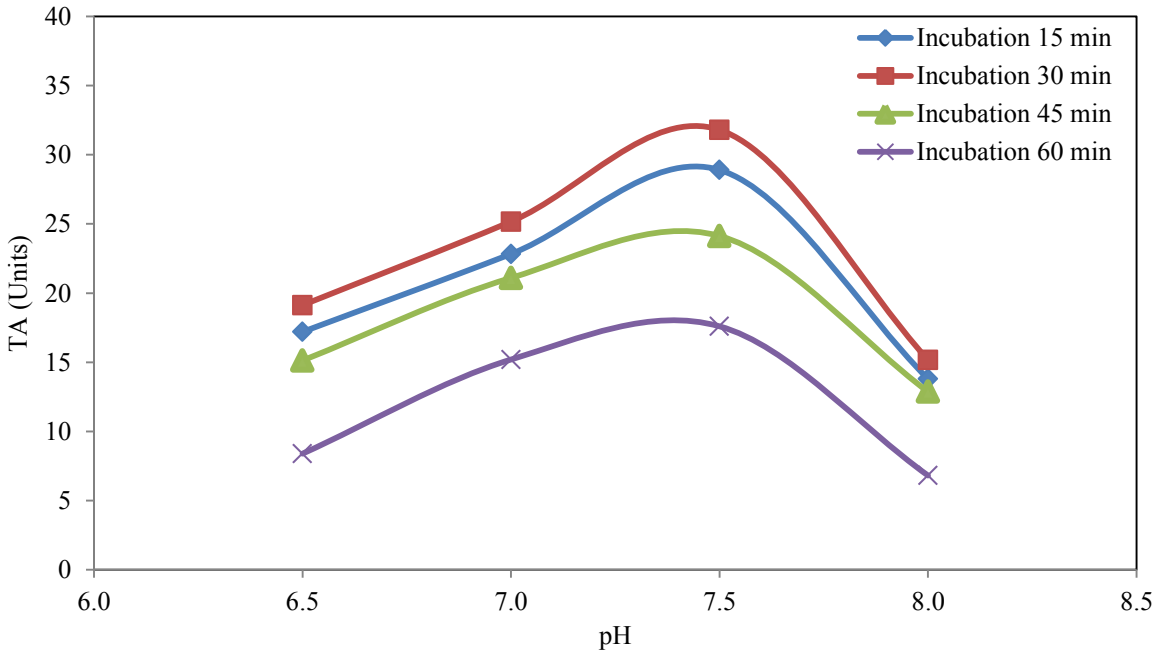


(b) 45-65% ammonium sulphate saturation

Figure 6.18. Effect of pH on the total activity (TA) of the cystatin extracted from the bovine lung at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3)

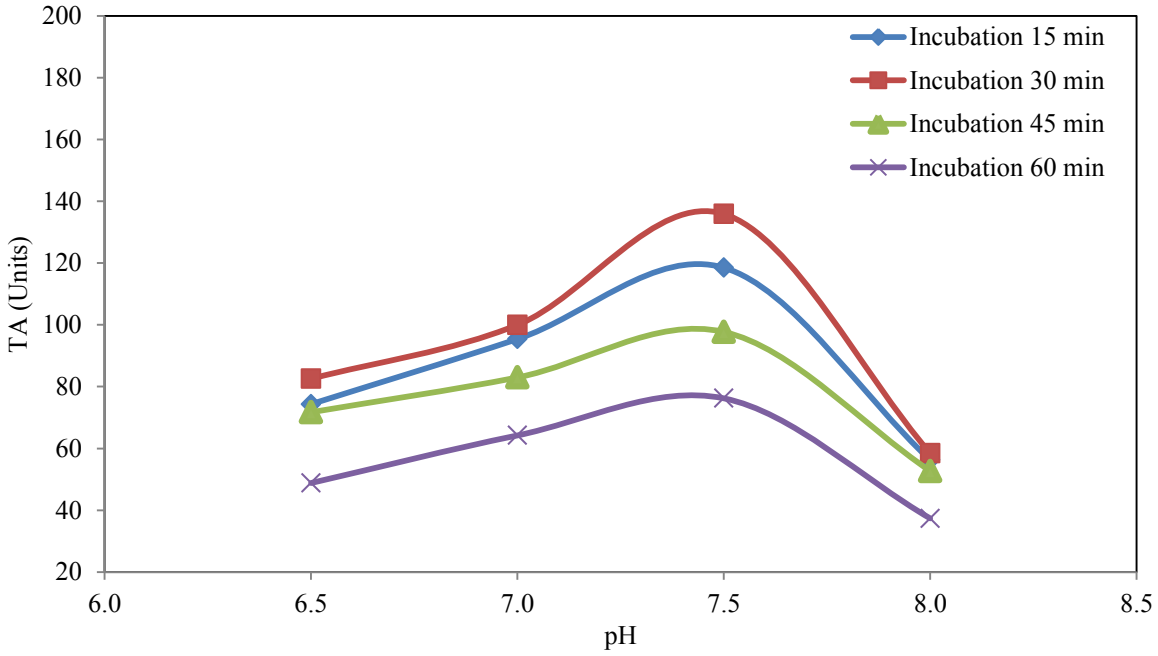


(a) 25-45% ammonium sulphate saturation

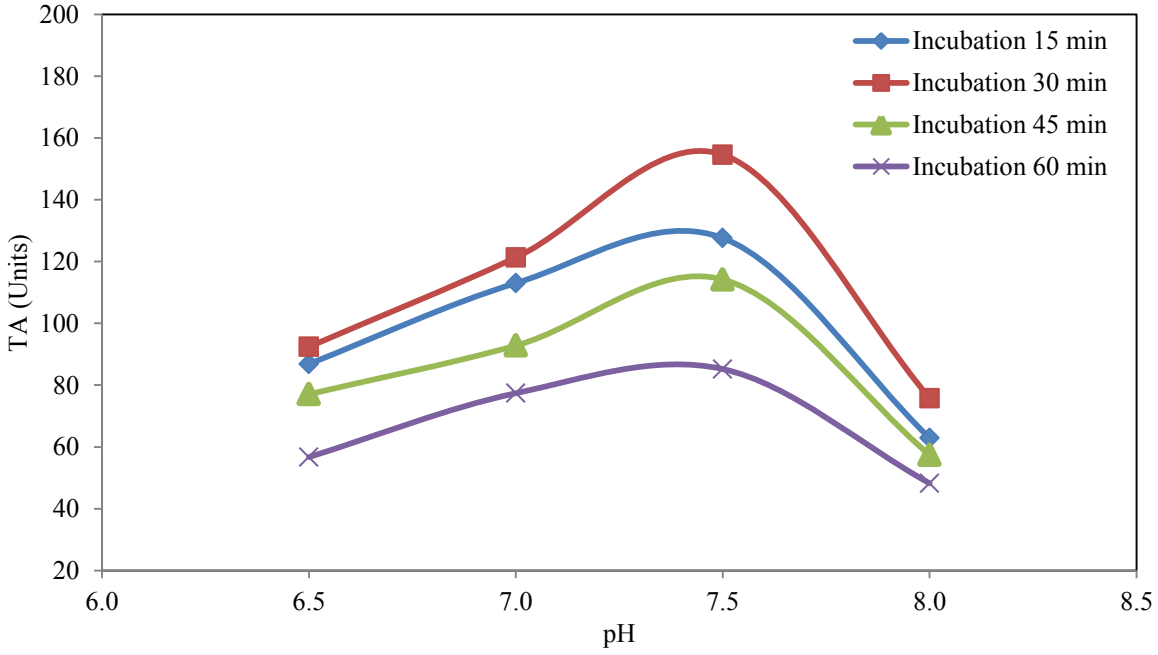


(b) 45-65% ammonium sulphate saturation

Figure 6.19. Effect of pH on the total activity (TA) of the cystatin extracted from the bovine pancreas at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3).

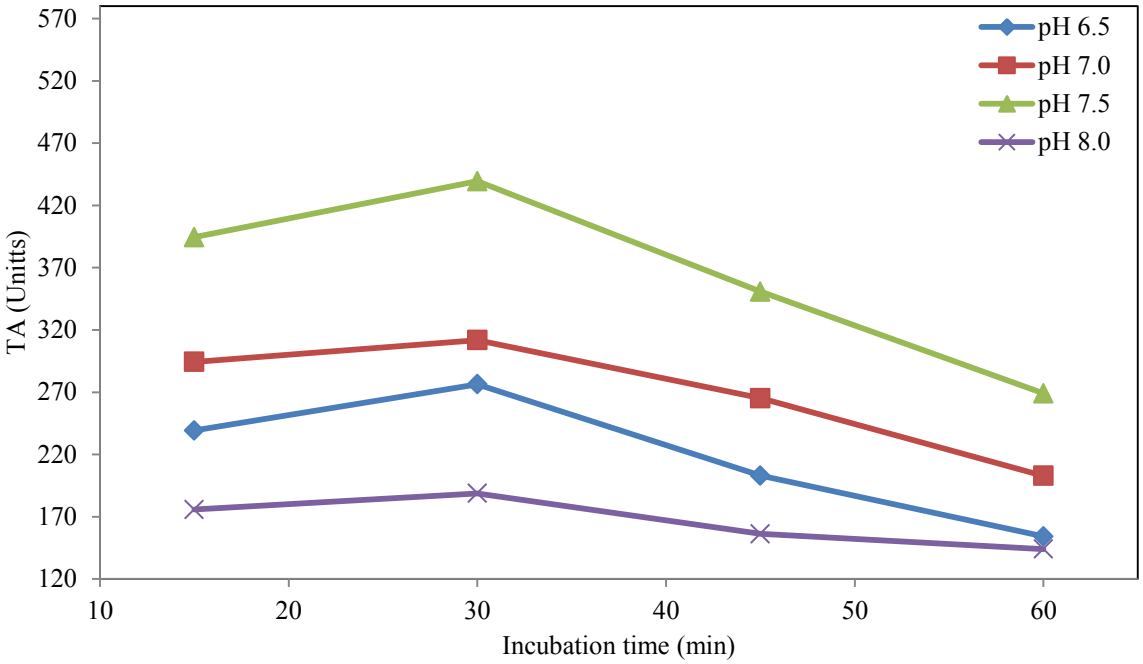


(a) 25-45% ammonium sulphate saturation

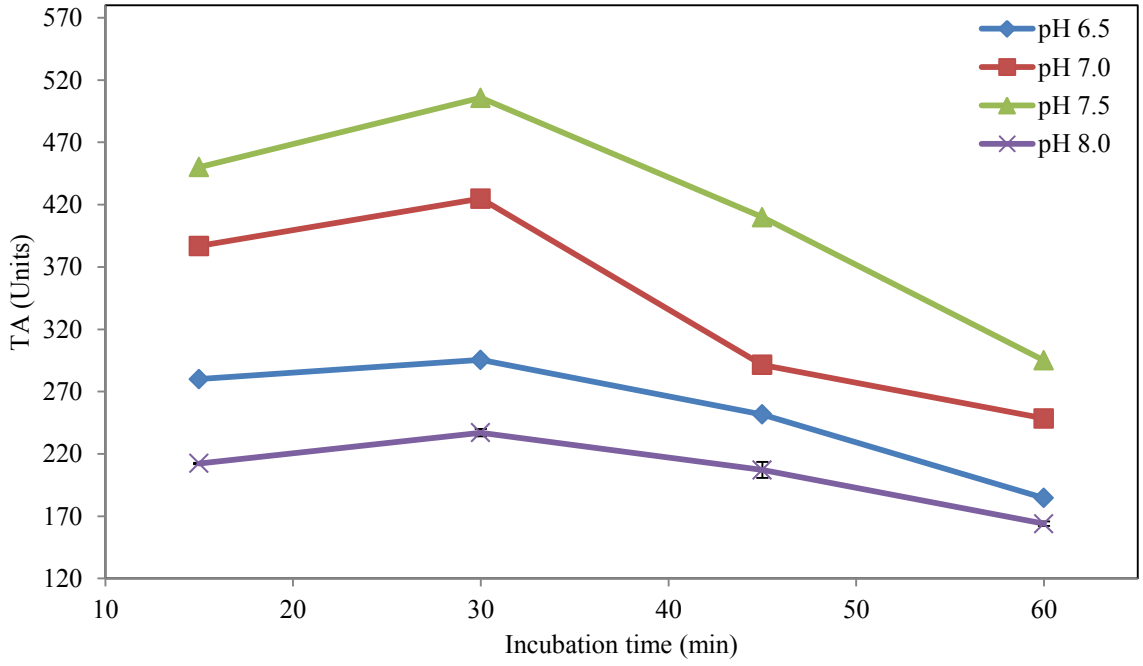


(b) 45-65% ammonium sulphate saturation

Figure 6.20. Effect of pH on the total activity (TA) of the cystatin extracted from the bovine lung and pancreas mixture at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3).

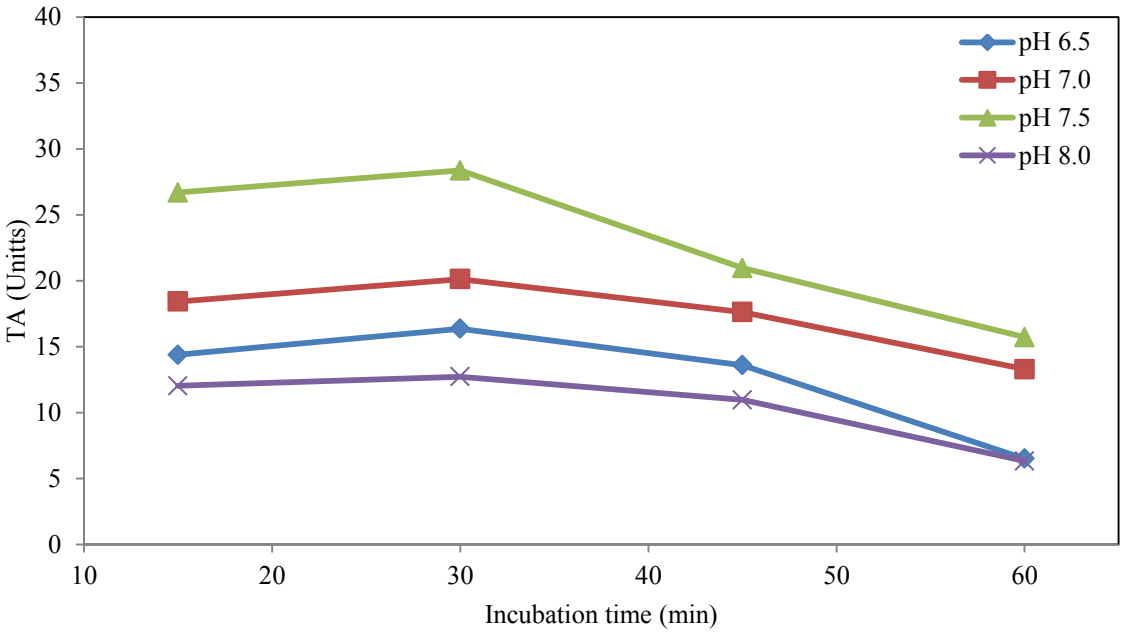


(a) 25-45% ammonium sulphate saturation

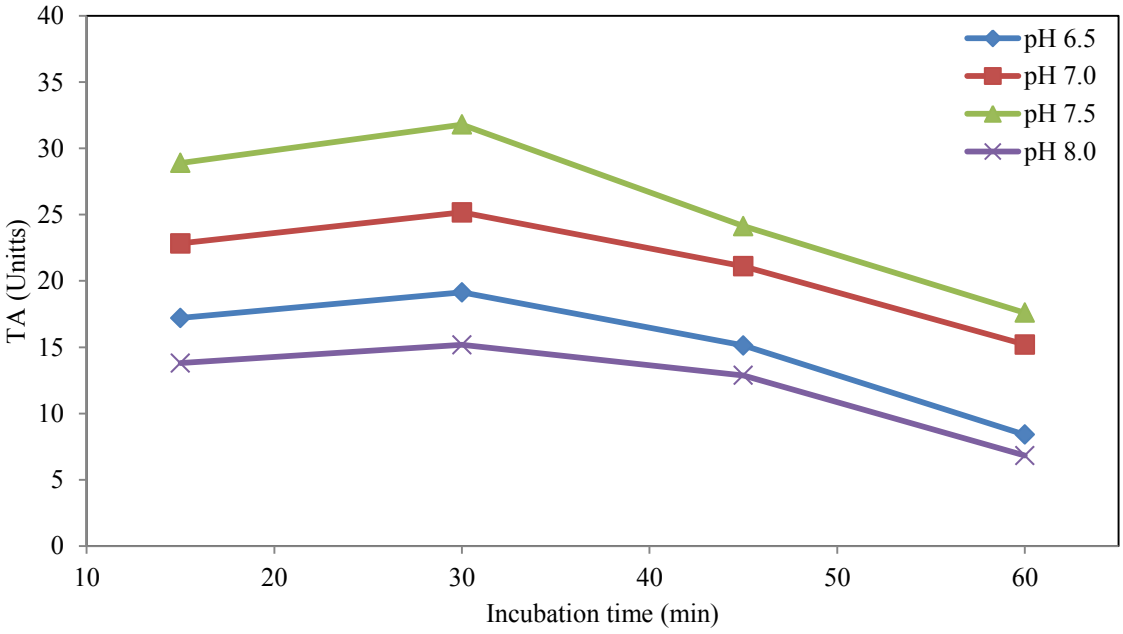


(a) 25-45% ammonium sulphate saturation

Figure 6.21. Effect of incubation time on the total activity (TA) of the cystatin extracted from the bovine lung at different pH and ammonium sulphate saturation levels (mean ± std, n=3).

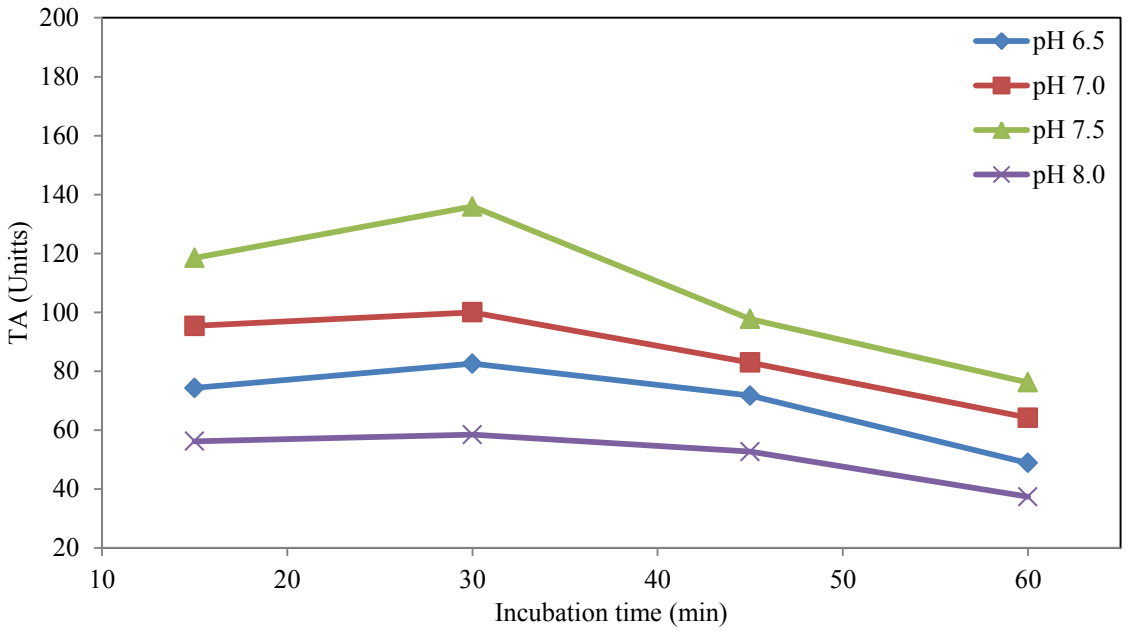


(a) 25-45% ammonium sulphate saturation

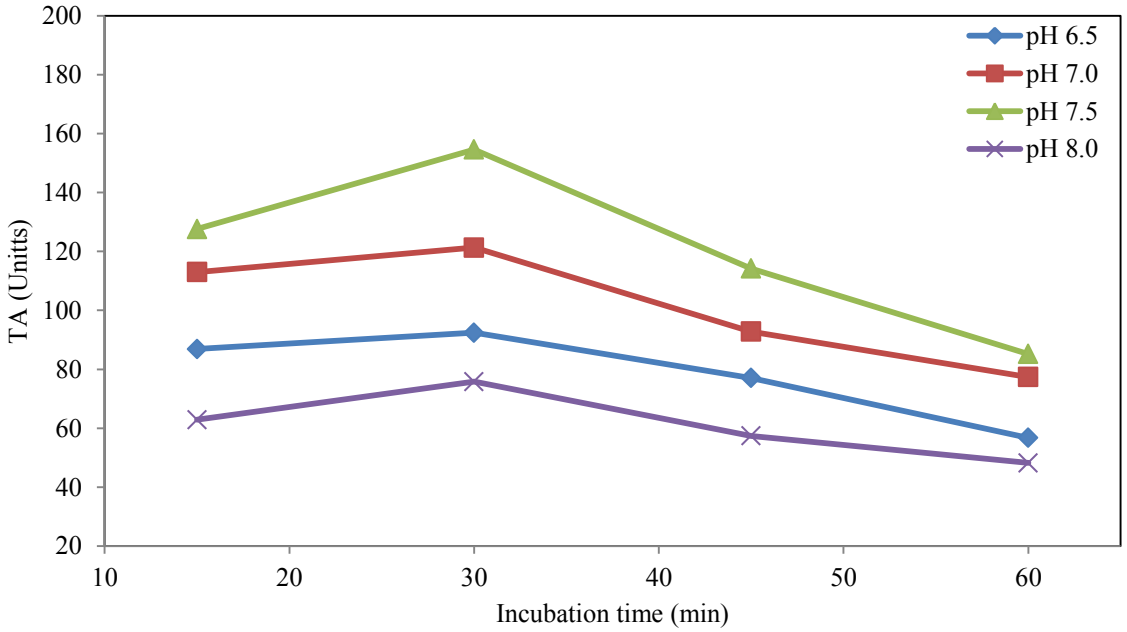


(b) 45-65% ammonium sulphate saturation

Figure 6.22. Effect of incubation time on the total activity (TA) of the cystatin extracted from the bovine pancreas at different pH and ammonium sulphate saturation levels (mean ± std, n=3).



(a) 25-45% ammonium sulphate saturation



(b) 45-65% ammonium sulphate saturation

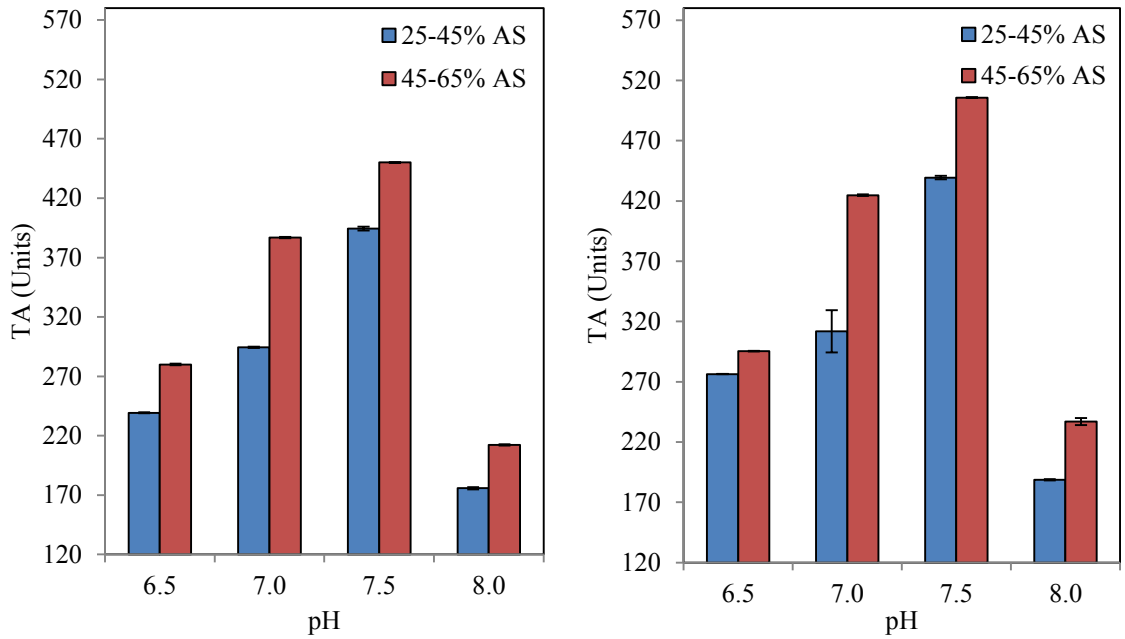
Figure 6.23. Effect of incubation time on the total activity (TA) of the cystatin extracted from the bovine lung and pancreas mixture at different pH and ammonium sulphate saturation levels (mean ± std, n=3).

time from 15to 30 min increased the TA of cystatin extracted from bovine lung sample from 239.3 to 276.4 Units (15.5%), from 294.3 to 311.8 Units (5.9%), from 394.4 to 439.4 Units (11.4%) and from 175.8 to 188.7 Units (7.3%) at the pH level 6.5, 7.0, 7.5 and 8.0, respectively. A further increase in the incubation time from 30 to 60 min decreased the TA from 276.4 to 154.2 Units, 311.8 to 202.9 Units, 439.4 to 269.0 Units and 188.7 to 144.0 Units at the pH levels of 6.5, 7.0, 7.5 and 8.0, respectively. Similar trends were observed with the TA of the cystatin extracted from the bovine pancreas and mixture of lungs and pancreas samples. All the three samples (bovine lung, pancreas and mixture of lungs and pancreas samples) also exhibited similar trends with the 45-65% ammonium sulphate saturation. The highest TA of cystatin was observed at the incubation time of 30 min for all samples at all pH levels.

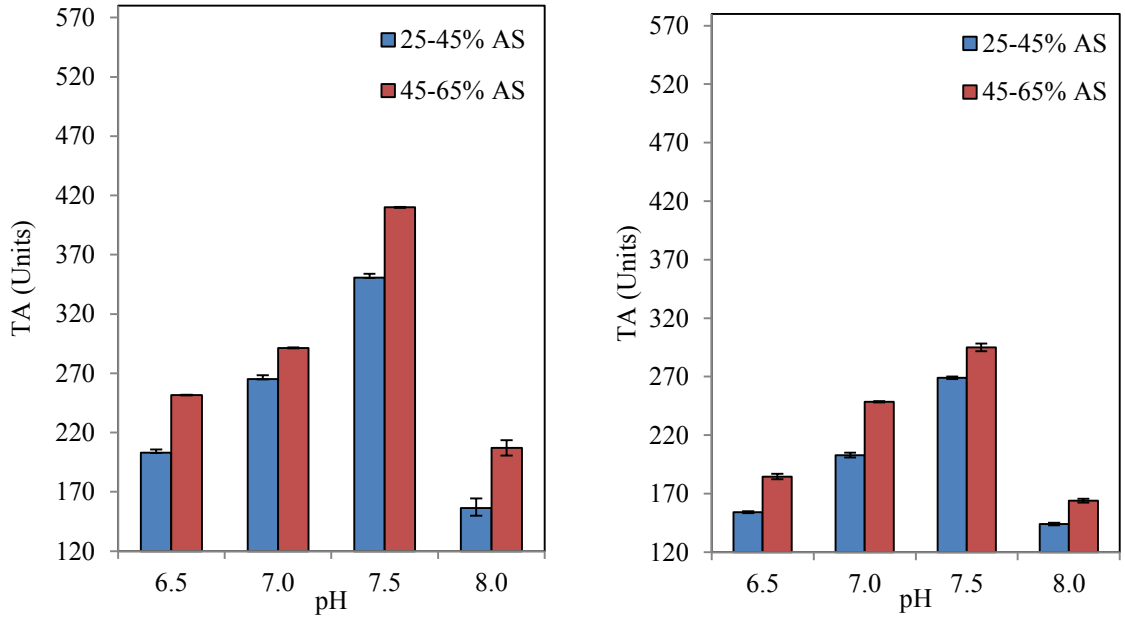
6.2.2.4. Effect of ammonium sulphate saturation on Total activity (TA): The effect of ammonium sulphate saturation (25-45% and 45-65%) on the total activity (TA) of the cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different pH (6.5, 7.0, 7.5, and 8.0) and incubation times (15, 30, 45 and 60 min) are shown in Figures 6.24-6.26. With the 15 min incubation, increasing the ammonium sulphate saturation from 25-45% to 45-65% increased the TA of the cystatin extracted from the bovine lung sample from 239.3 to 280 Units (17%), from 294.3 to 386.9 mg (31.4%), from 394.4 to 450.1 mg (14.1%) and from 175.8 to 212.3 mg (20.7%) at the pH levels of 6.5, 7.0, 7.5 and 8.0, respectively. Similar trends were observed for the TA of the cystatin extracted from the three samples (bovine lung, pancreas and mixture of lungs and pancreas samples) at the incubation times of 30, 45 and 60 min. Increasing the ammonium sulphate saturation level from 25-45% to 45-65% increased the TA of the cystatin.

6.2.3. Specific Activity (SA)

The effects of the sample type (bovine lung, pancreas and mixture of lung and pancreas), pH (6.5, 7.0, 7.5, and 8.0), incubation time (15, 30, 45 and 60 min) and ammonium sulphate saturation level (25-45% and 45-65%) on the specific activity (SA) of cystatin are shown in Tables 6.12 - 6.14. The specific activity (SA) of the cystatin extracted from

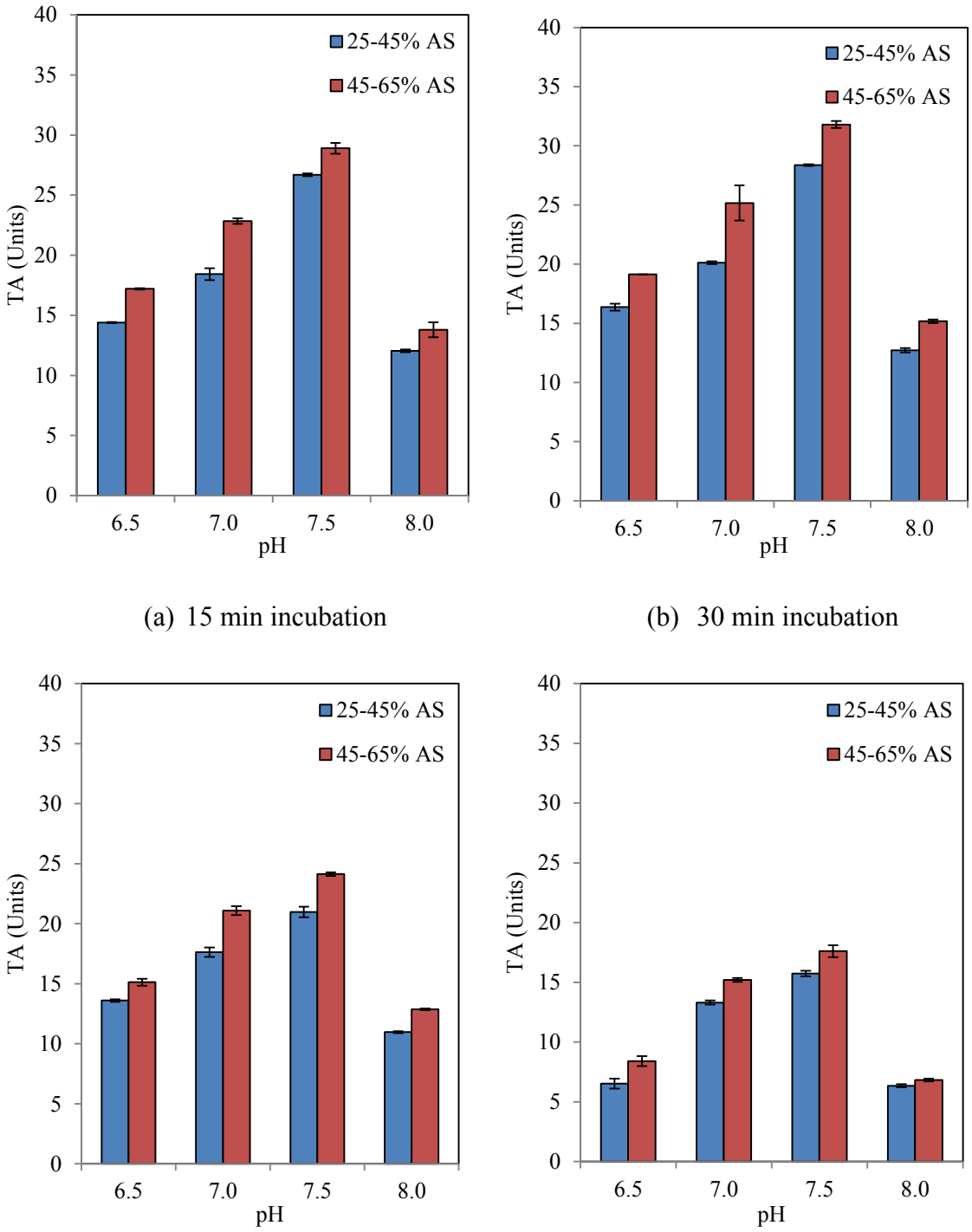


(a) 15 min incubation (b) 30 min incubation



(c) 45 min incubation (d) 60 min incubation

Figure 6.24. Effect of ammonium sulphate (AS) saturation on the total activity (TA) of the cystatin extracted from the bovine lung at different pH levels and incubation times (mean ± std, n=3).



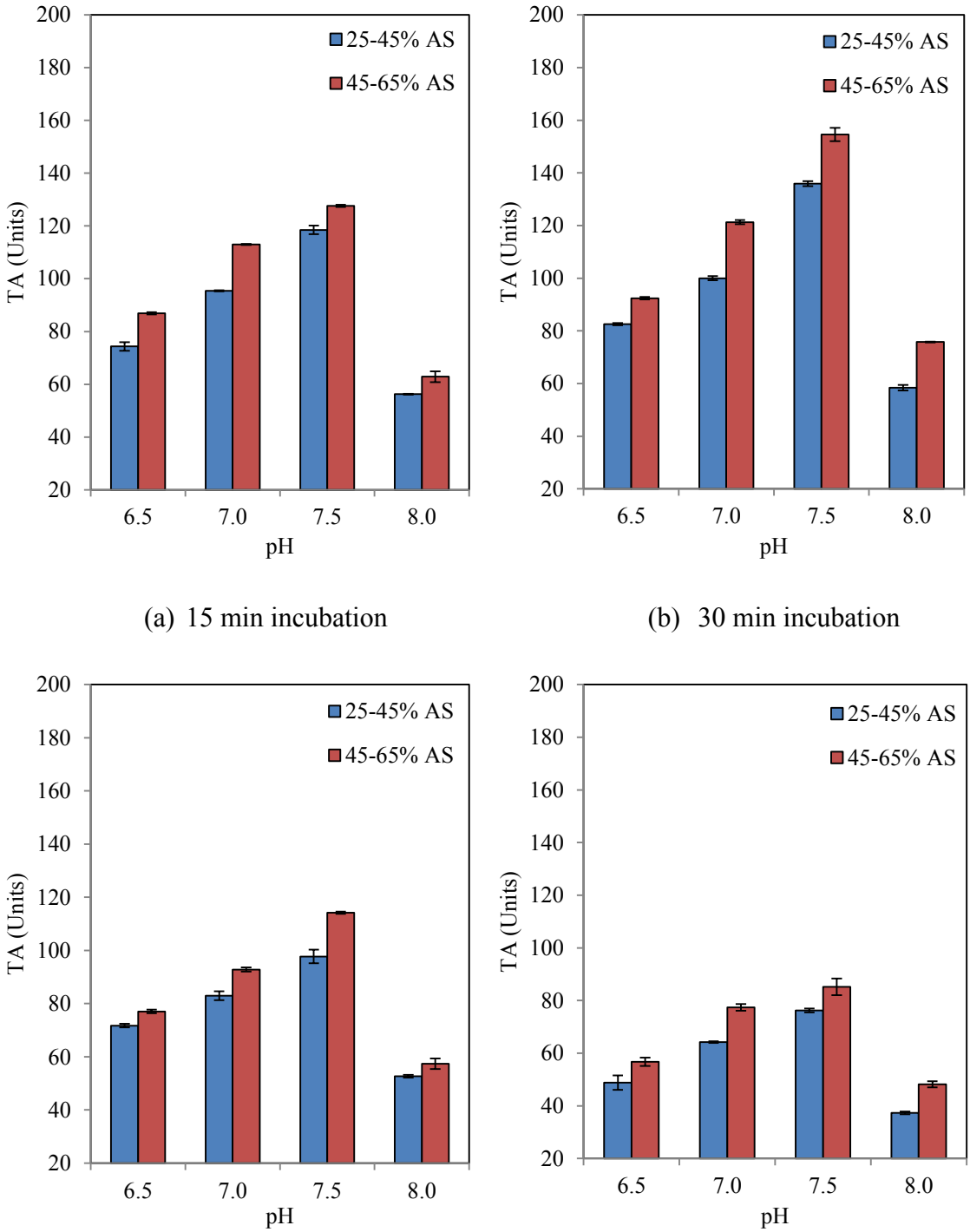
(a) 15 min incubation

(b) 30 min incubation

(c) 45 min incubation

(d) 60 min incubation

Figure 6.25. Effect of ammonium sulphate (AS) saturation on the total activity (TA) of the cystatin extracted from the bovine pancreas at different pH levels and incubation times (mean ± std, n=3).



(a) 15 min incubation

(b) 30 min incubation

(c) 45 min incubation

(d) 60 min incubation

Figure 6.26. Effect of ammonium sulphate (AS) saturation on the total activity (TA) of the cystatin extracted from the bovine lung and pancreas mixture at different pH levels and incubation times (mean ± std, n=3).

the bovine lung, pancreas and mixture of lung and pancreas varied from 0.090 to 0.112 Units/mg, 0.007 to 0.006 Units/mg and 0.033 to 0.030 Units/mg, respectively.

Analysis of variance was performed on the specific activity data using Minitab (Version 16.2.3, Minitab Inc, State College, Pennsylvania, USA). The results are shown in Table 6.19. The effects of sample types, pH, incubation times and ammonium sulphate saturation levels were significant at the 0.001 level. The two, three and four way interactions between the parameters were also significant at the 0.001 level.

The Tukey grouping was also performed on the specific activity (SA) data as shown in Table 6.20. The bovine lung, pancreas and mixture of lung and pancreas samples were significantly different from each other at the 0.05 level. The highest average specific activity (SA) of cystatin of 0.129010 Units/mg was obtained from the bovine lung sample. All the incubation times were significantly different from each other at the 0.05 level. The highest average specific activity (SA) of cystatin of 0.067389 was obtained at 30 min incubation. All pH levels were significantly different from each other at the 0.05 level. The highest average specific activity (SA) of cystatin of 0.072139 Units/mg was obtained the pH 7.5. The ammonium sulphate saturation levels were also significantly different from each other at the 0.05 level. The highest average specific activity (SA) of cystatin of 0.063965 Units/mg was obtained with the 45-65% ammonium sulphate saturation.

6.2.3.1. Effect of Sample Type on Specific Activity (SA): The effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on specific activity (SA) of cystatin extracted at different incubation times (15, 30, 45 and 60 min) and ammonium sulphate saturation levels (25-45% and 45-65%) are shown in Figures 27 - 30. With the 25-45 % saturation, pH 6.5 and 15 min incubation, the specific activity (SA) of the cystatin extracted from the bovine lung was 0.090 Units/mg, which was higher than those extracted from the pancreas and mixture of lung and pancreas samples All three samples (bovine lung, pancreas and mixture of lung and pancreas) exhibited similar trends with the 45-65% ammonium sulphate saturation at all pH levels and incubation

Table 6.19. Analysis of variance for specific activity (SA) - ammonium sulphate extraction

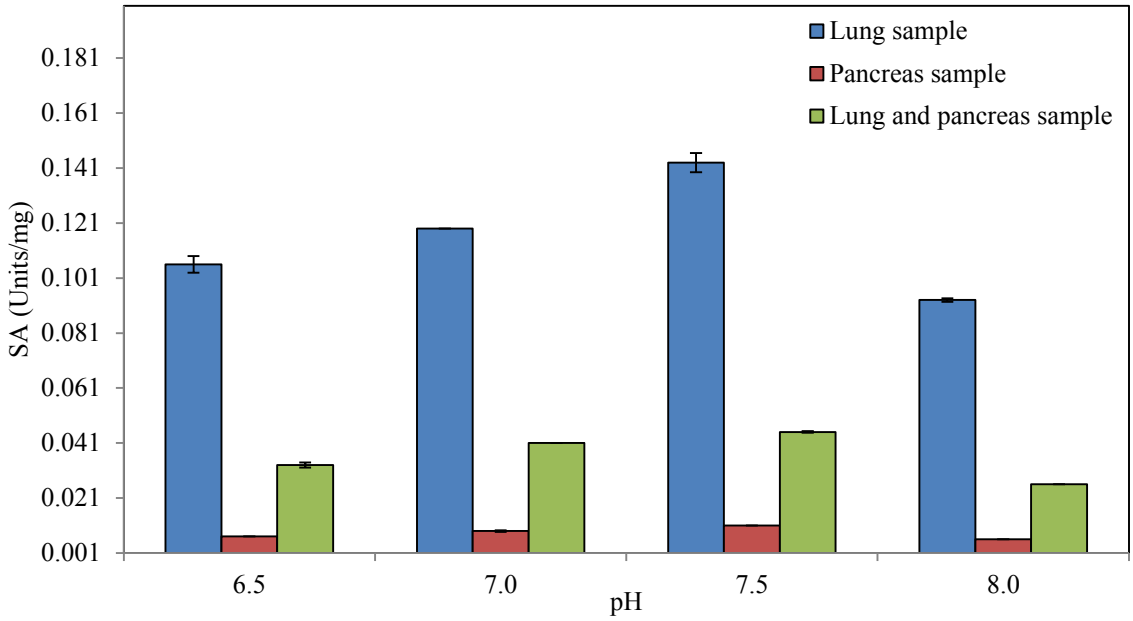
Source	DF	SS	MS	F	P
Total	287	0.793107			
Model					
ST	2	0.728604	0.008899	1595.2	0.001
IT	3	0.010841	0.364302	65304.3	0.001
PH	3	0.021704	0.003614	647.8	0.001
SL	1	0.003120	0.007235	1296.9	0.001
ST*IT	6	0.008031	0.003120	559.4	0.001
ST*PH	6	0.011419	0.001338	239.9	0.001
ST*SL	2	0.002217	0.001903	341.2	0.001
IT*PH	9	0.001242	0.001108	198.7	0.001
IT*SL	3	0.000246	0.000138	24.7	0.001
PH*SL	3	0.000456	0.000082	14.7	0.001
ST*IT*PH	18	0.002135	0.000119	27.3	0.001
ST*IT*SL	6	0.000751	0.000125	22.4	0.001
IT*PH*SL	9	0.000551	0.000061	11.0	0.001
ST*IT*PH*SL	18	0.000684	0.000038	6.8	0.001
Error	198	0.001105	0.000006		

ST : Sample type
IT : Incubation time (min)
PH : pH
SL : Saturation level (%)
DF : Degree of freedom
SS : Sum of square
MS : Mean of square
R^2 : 0.9977
CV : 8.6%

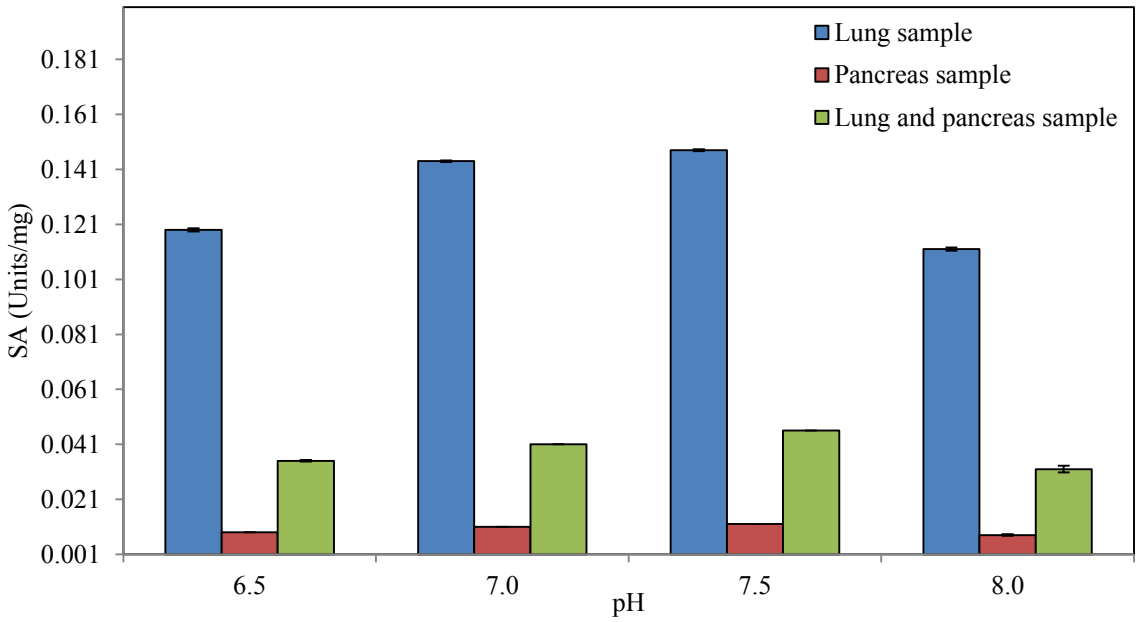
Table 6.20. Tukey grouping for specific activity (SA)- ammonium sulphate extraction

Factors	Level	N	Mean SA (Units/mg)	Tukey Grouping
Samples	Lung sample	96	0.129010	A
	Lung and pancreas sample	96	0.043604	B
	Pancreas sample	96	0.009406	C
Incubation time (min)	30	72	0.067389	A
	45	72	0.065611	B
	15	72	0.057417	C
	60	72	0.052278	D
pH	7.5	72	0.072139	A
	7.0	72	0.065181	B
	6.5	72	0.056014	C
	8.0	72	0.049361	D
Saturation (%)	45-65%	144	0.063965	A
	25-45%	144	0.057382	B

Means share the letter are not significantly different from one another at the 0.05 level.

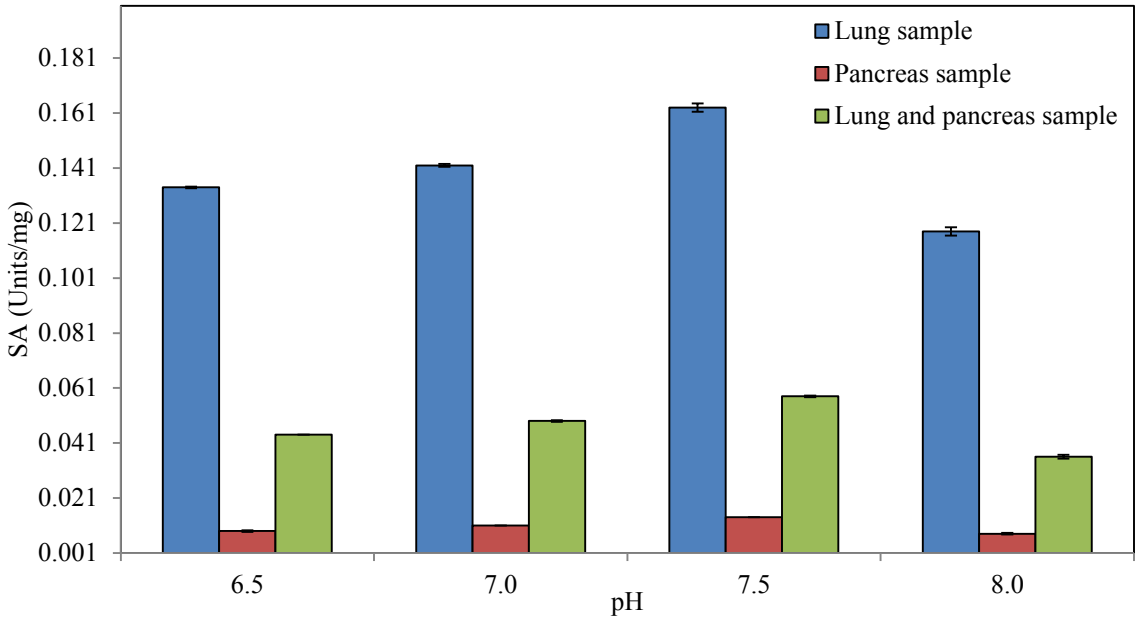


(a) 25-45% ammonium sulphate saturation

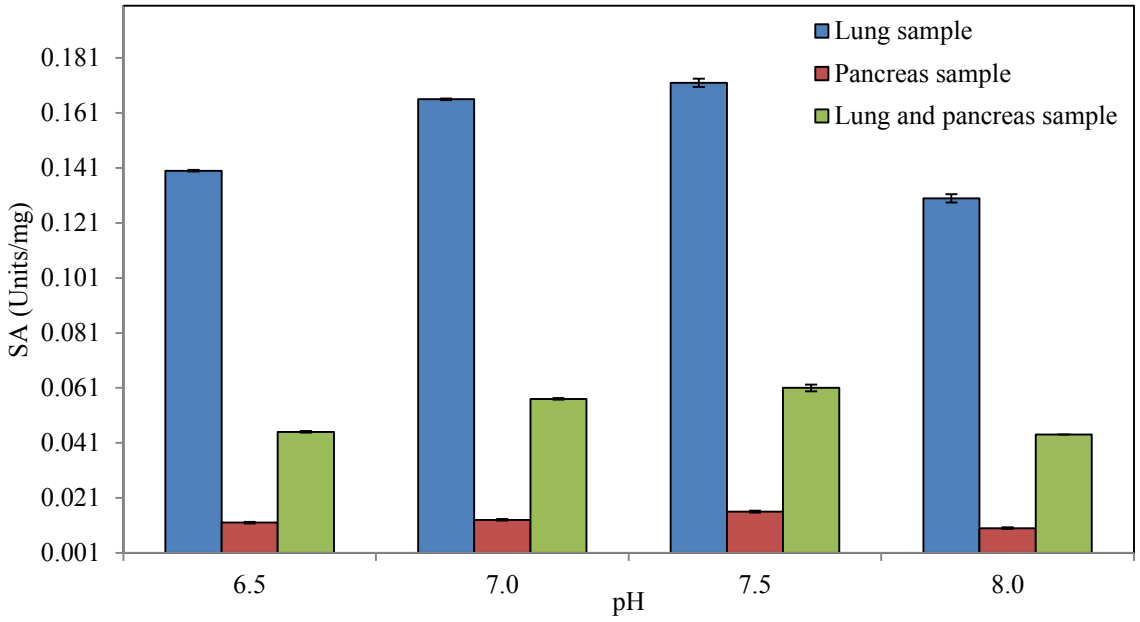


(b) 45-65% ammonium sulphate saturation

Figure 6.27. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on the specific activity (SA) of the cystatin extracted at 15 min incubation and different levels of pH and ammonium sulphate saturation

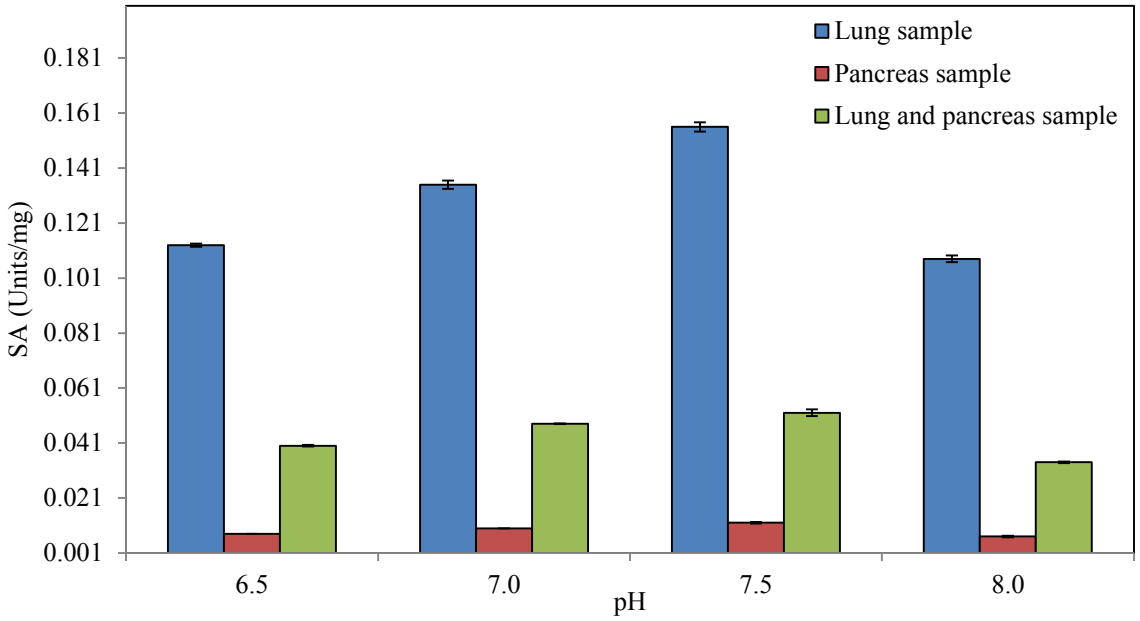


(a) 25-45% ammonium sulphate saturation

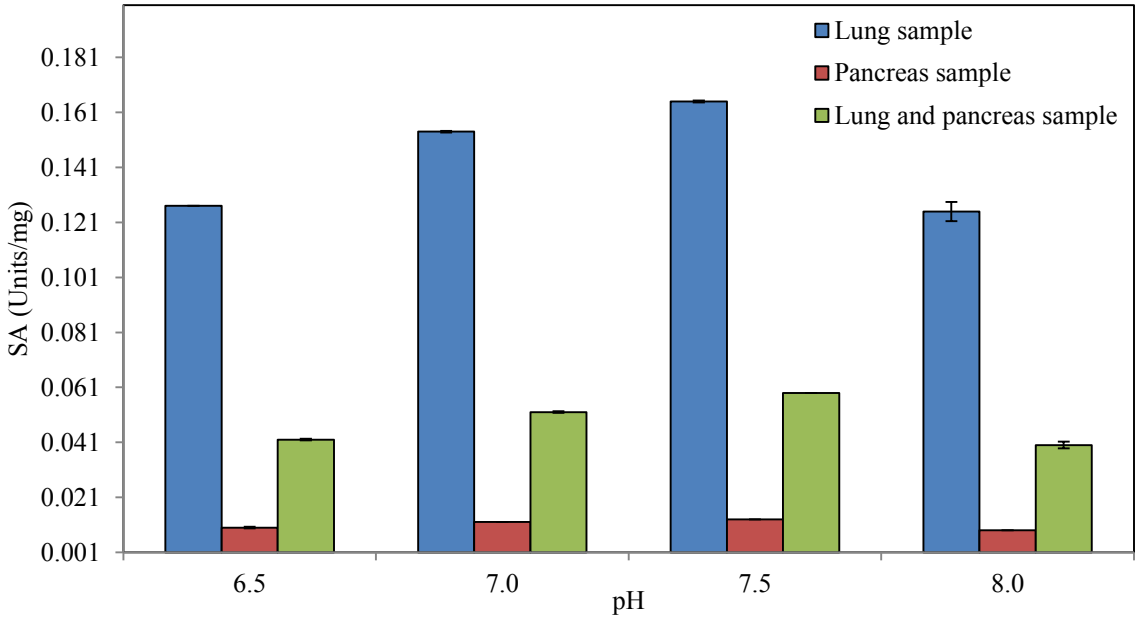


(b) 45-65% ammonium sulphate saturation

Figure 6.28. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on specific activity (SA) of the cystatin extracted at 30 min incubation and different levels of pH and ammonium sulphate saturation

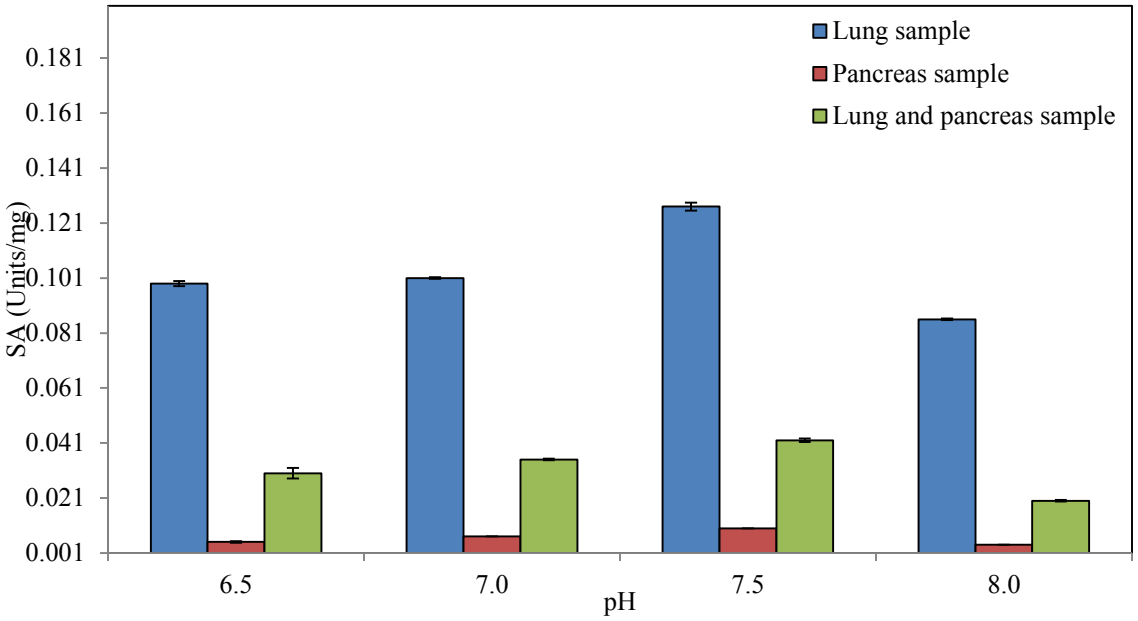


(a) 25-45% ammonium sulphate saturation

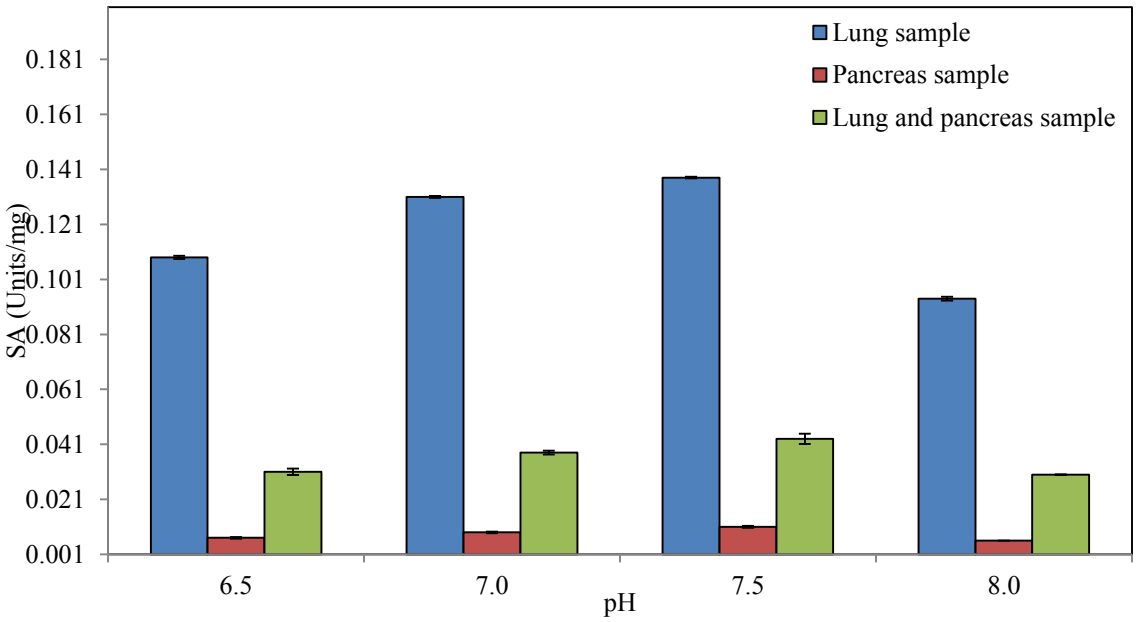


(b) 45-65% ammonium sulphate saturation

Figure 6.29. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on specific activity (SA) of the cystatin extracted at 45 min incubation and different levels of pH and ammonium sulphate saturation



(a) 25-45% ammonium sulphate saturation



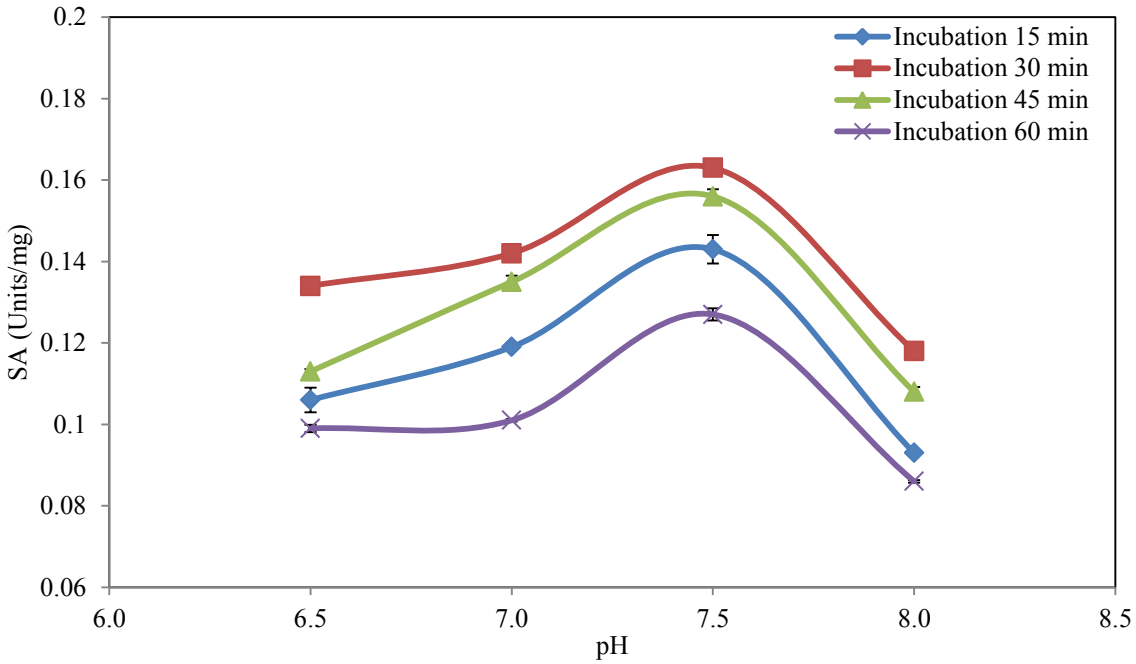
(b) 45-65% ammonium sulphate saturation

Figure 6.30. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on specific activity (SA) of the cystatin extracted at 60 min incubation and different levels of pH and ammonium sulphate saturation

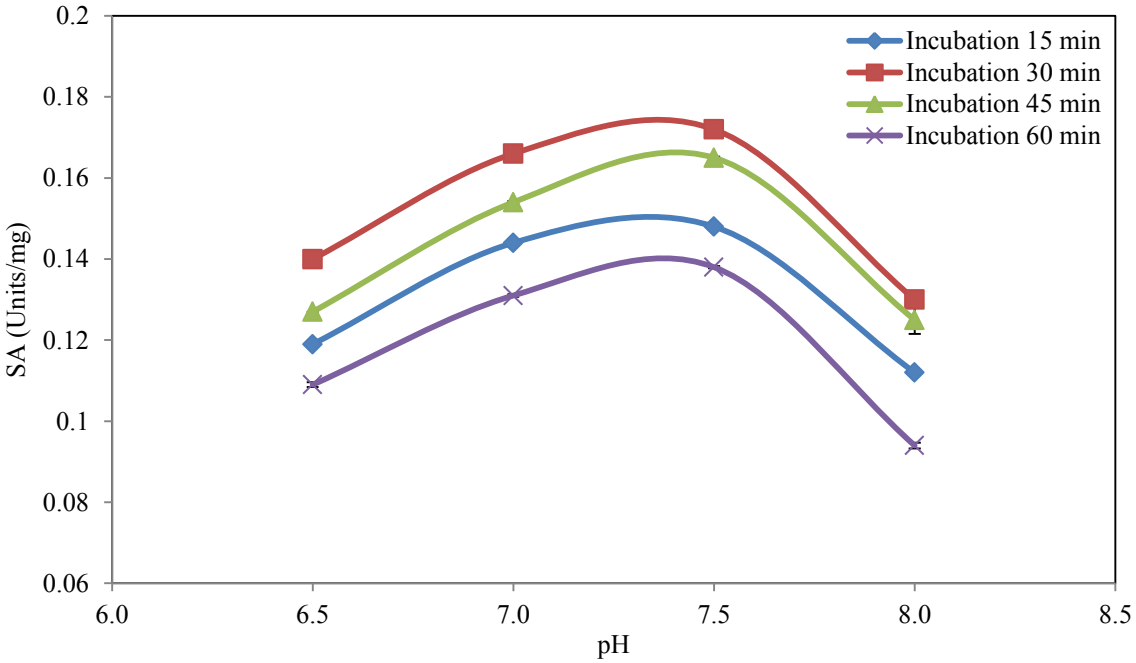
times. The bovine lung sample showed higher specific activity (SA) of cystatin than those of the pancreas and mixture of lung and pancreas samples under all conditions.

6.2.3.2. Effect of pH on Specific Activity (SA): The effect of pH (6.5, 7.0, 7.5, and 8.0) on specific activity (SA) of the cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different incubation times (15, 30, 45 and 60 min) and saturation levels (25-45% 45-65%) were shown in Figures 6.31 - 6.33. The SD for the graphs are too small (<2). With 25-45% ammonium sulphate saturation, increasing the pH from 6.5 to 7.5 increases the TP of the cystatin extracted from the bovine lung sample from 0.106 to 0.143 Units/mg (34.9%), from 0.134 to 0.163 Units/mg (21.6%), from 0.113 to 0.156 Units/mg (38%) and from 0.099 to 0.127 Units/mg (28.2%) for the incubation times of 15, 30, 45 and 60 min, respectively. A further increase in the pH from 7.5 to 8.0 decreased the SA of the cystatin from 0.143 to 0.093 Units/mg (34.9%), from 0.163 to 0.118 Units/mg (27.60%), from 0.156 to 0.108 Units/mg (30.7%) and from 0.127 to 0.086 Units/mg (32.2%) for the incubation times of 15, 30, 45 and 60 min, respectively. Similar trends were observed for the bovine pancreas and the mixture of lung and pancreas samples. All the three samples (bovine lung, pancreas and mixture of lung and pancreas samples) also exhibited similar trends with the 45-65% ammonium sulphate saturation at all incubation times. The pH 7.5 produced the highest SA of the cystatin extracted from all samples at all incubation times.

6.2.3.3. Effect of incubation time on Specific Activity (SA): The effect of incubation time (15, 30, 45 and 60 min) on the specific activity (SA) of the cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different pH levels (6.5, 7.0, 7.5, and 8.0) and ammonium sulphate saturation levels (25-45% and 45-65%) are shown in Figures 6.34 - 6.36. With the 25-45% ammonium sulphate saturation, increasing the incubation time from 15 to 30 min increased the SA of the cystatin extracted from the bovine lung sample from 0.106 to 0.134 Units/mg (26.4%), from 0.119 to 0.142 Units/mg (19.3%), from 0.143 to 0.163 Units/mg (13.9%) and from 0.093 to 0.118 Units/mg (26.8%) at the pH levels of 6.5, 7.0, 7.5 and 8.0, respectively. A further increase in the incubation time from 30 to 60 min decreased the SA of the cystatin from 0.134 to 0.099 Units/mg (26.11%), from 0.142 to 0.101 Units/mg (28.8%), from

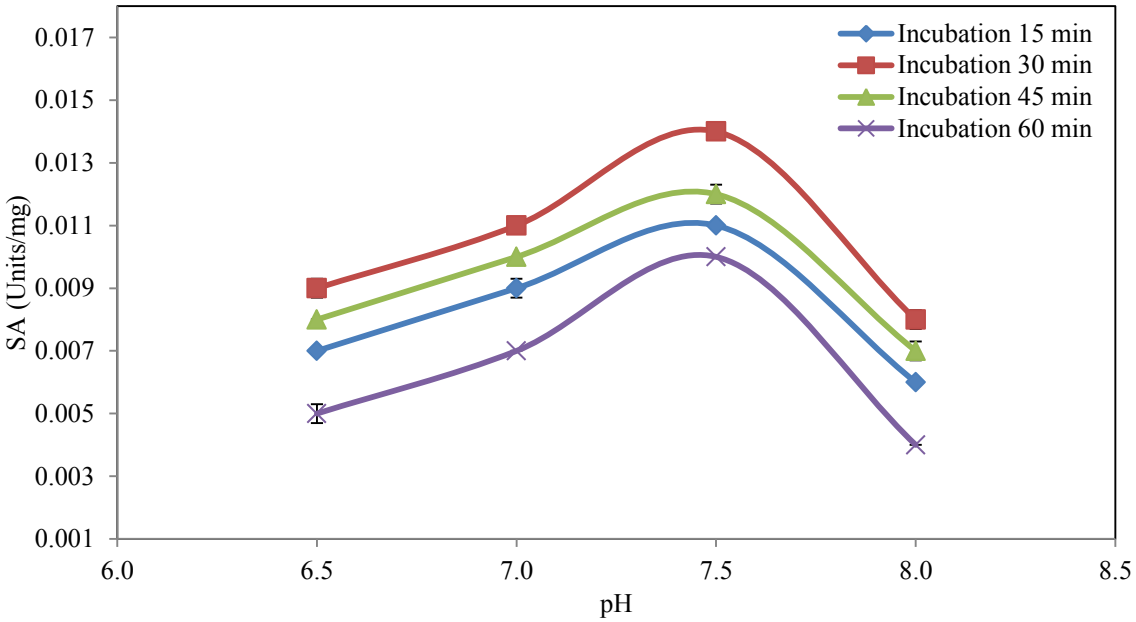


(a) 25-45% ammonium sulphate saturation

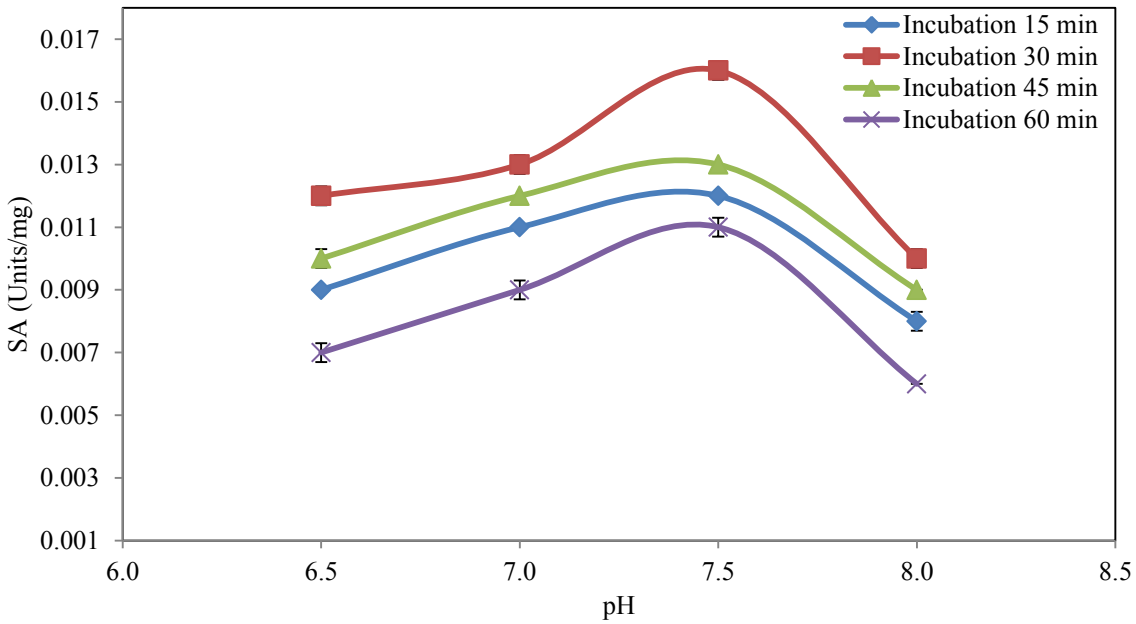


(b) 45-65% ammonium sulphate saturation

Figure 6.31. Effect of pH on the specific activity (SA) of the cystatin extracted from the bovine lung at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3).

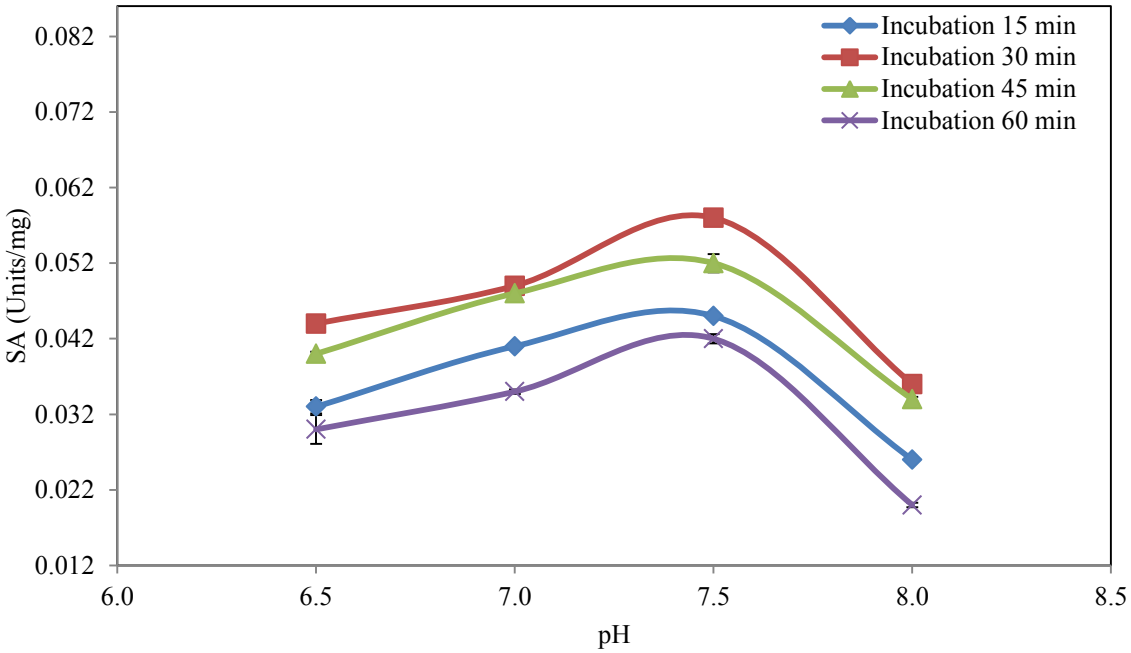


(a) 25-45% ammonium sulphate saturation

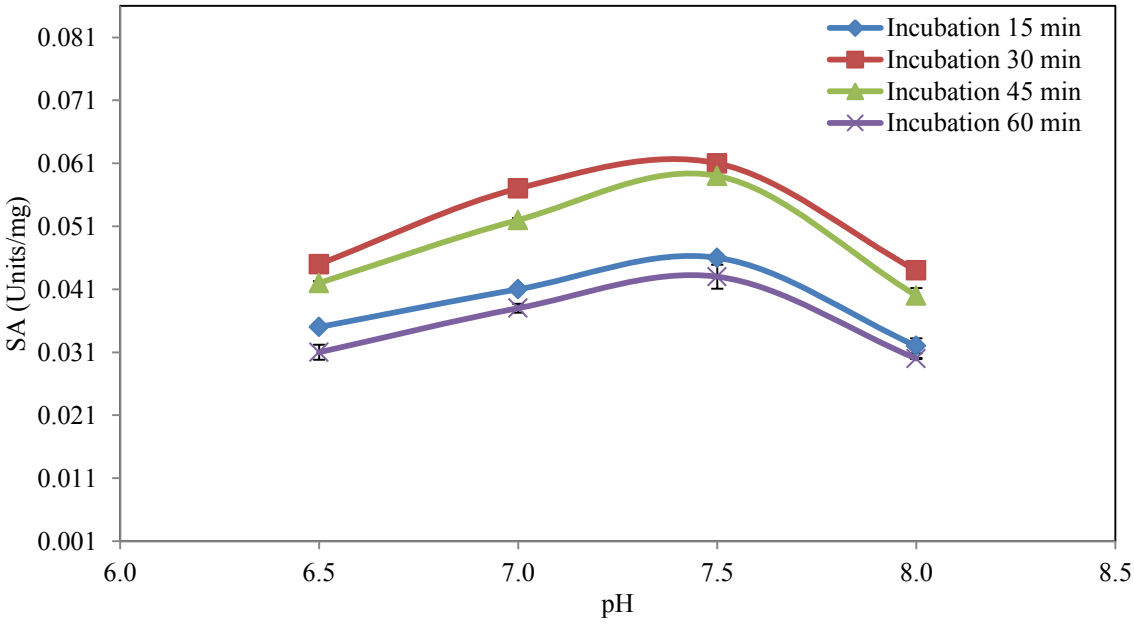


(b) 45-65% ammonium sulphate saturation

Figure 6.32. Effect of pH on the specific activity (SA) of the cystatin extracted from the bovine pancreas at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3).

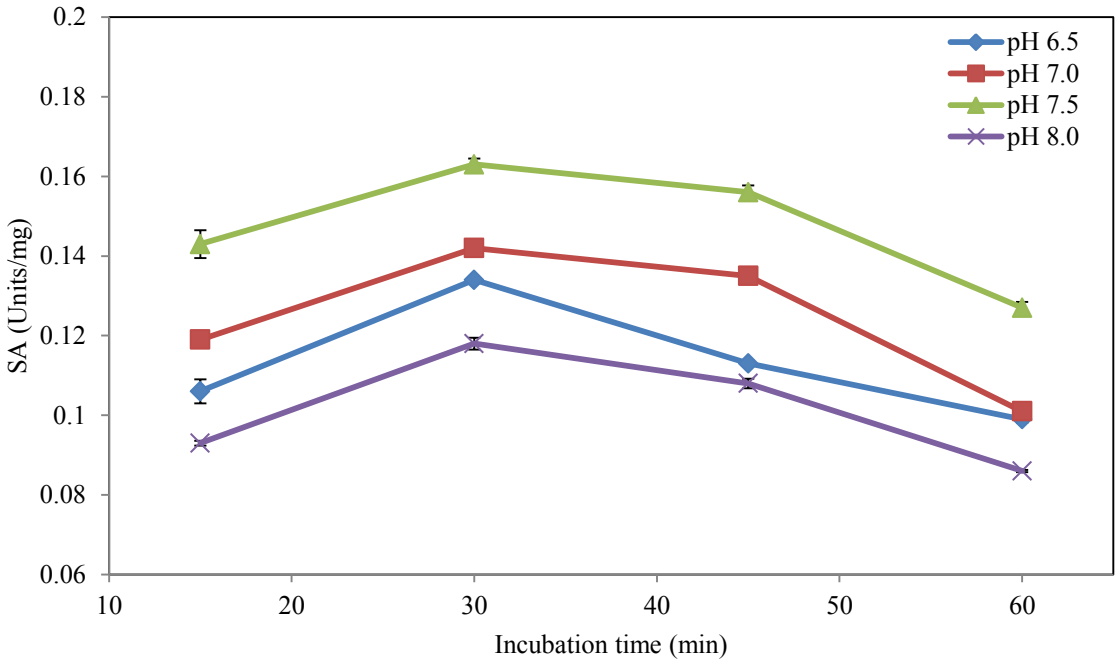


(a) 25-45% ammonium sulphate saturation

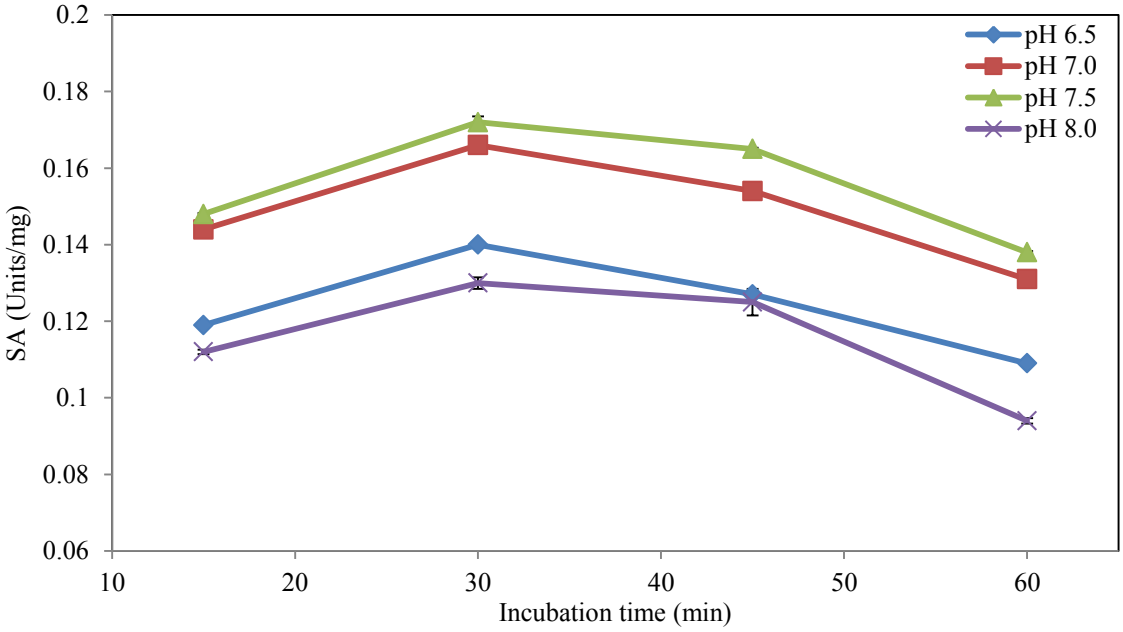


(b) 45-65% ammonium sulphate saturation

Figure 6.33. Effect of pH on the specific activity (SA) of the cystatin extracted from the bovine lung and pancreas mixture at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3).

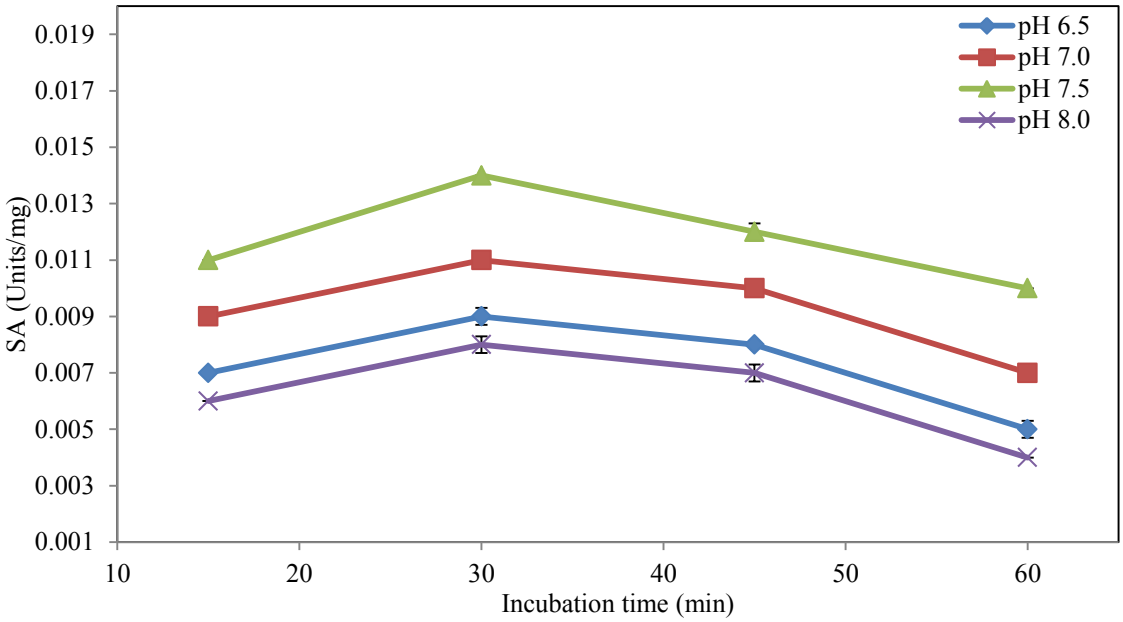


(a) 25-45% ammonium sulphate saturation

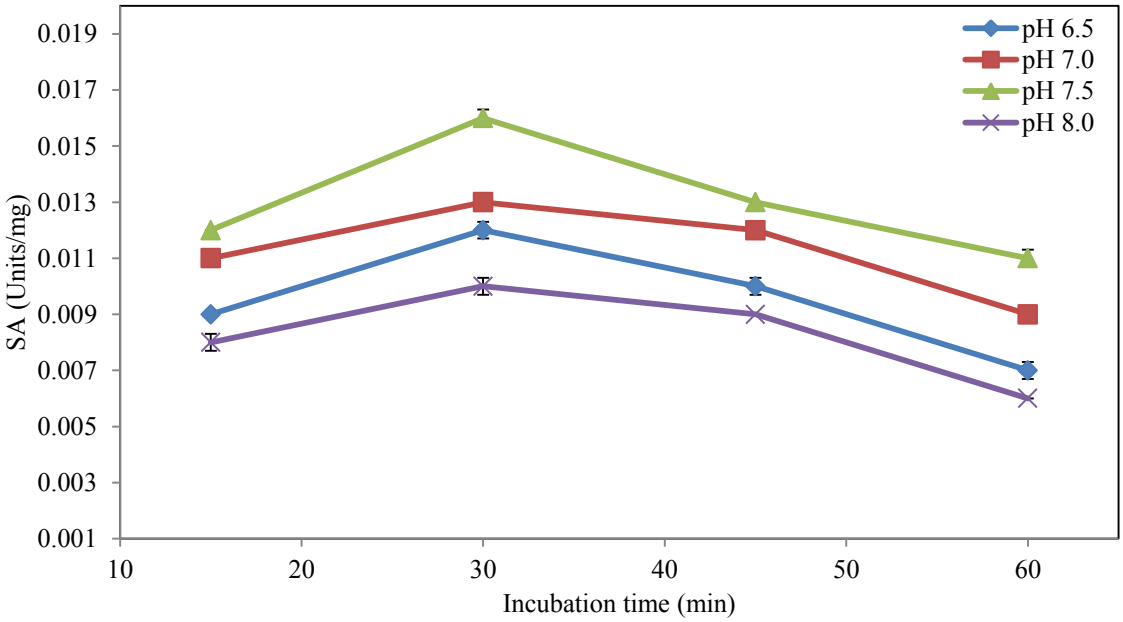


(b) 45-65% ammonium sulphate saturation

Figure 6.34. Effect of incubation time on the specific activity (SA) of the cystatin extracted from the bovine lung at different pH and ammonium sulphate saturation levels (mean ± std, n=3).

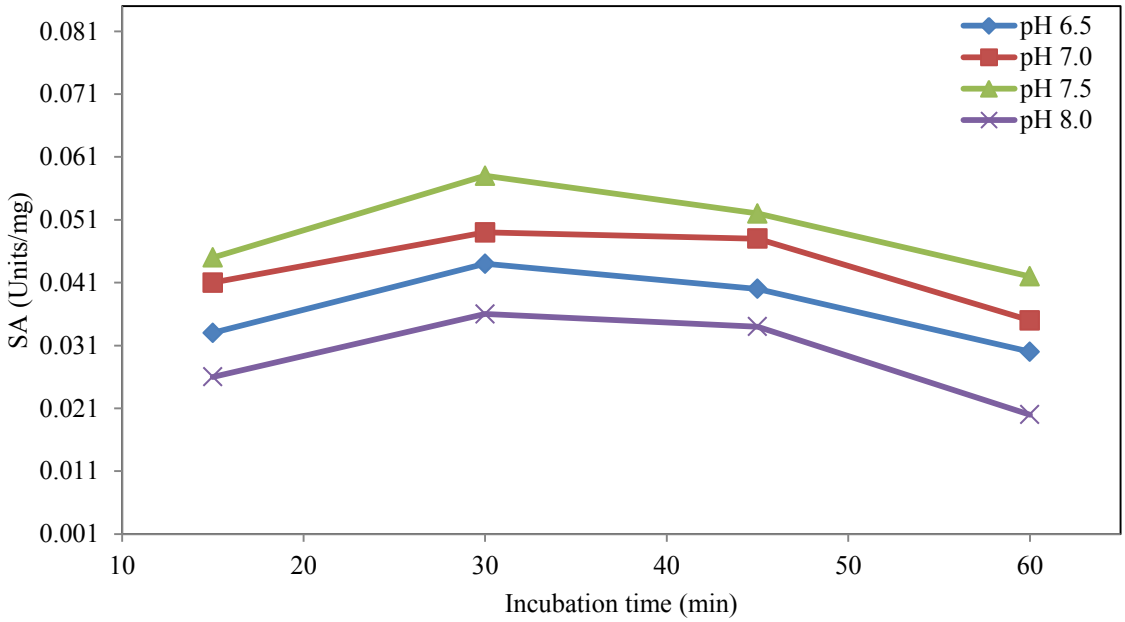


(a) 25-45% ammonium sulphate saturation

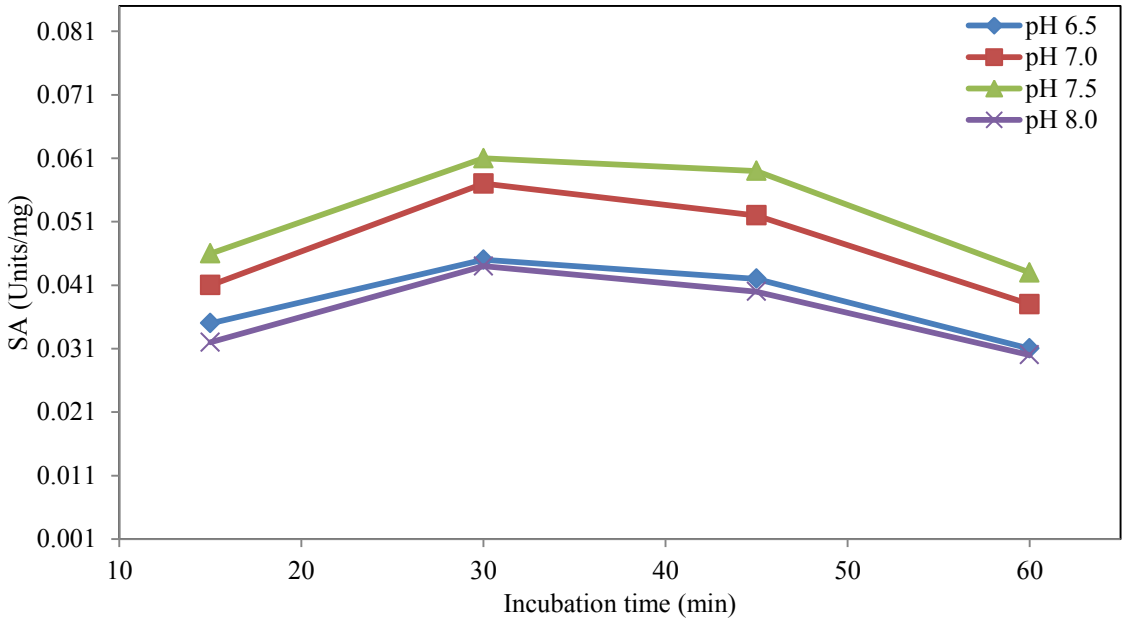


(b) 45-65% ammonium sulphate saturation

Figure 6.35. Effect of incubation time on the specific activity (SA) of the cystatin extracted from the bovine pancreas at different pH and ammonium sulphate saturation levels (mean ± std, n=3).



(a) 25-45% ammonium sulphate saturation



(b) 45-65% ammonium sulphate saturation

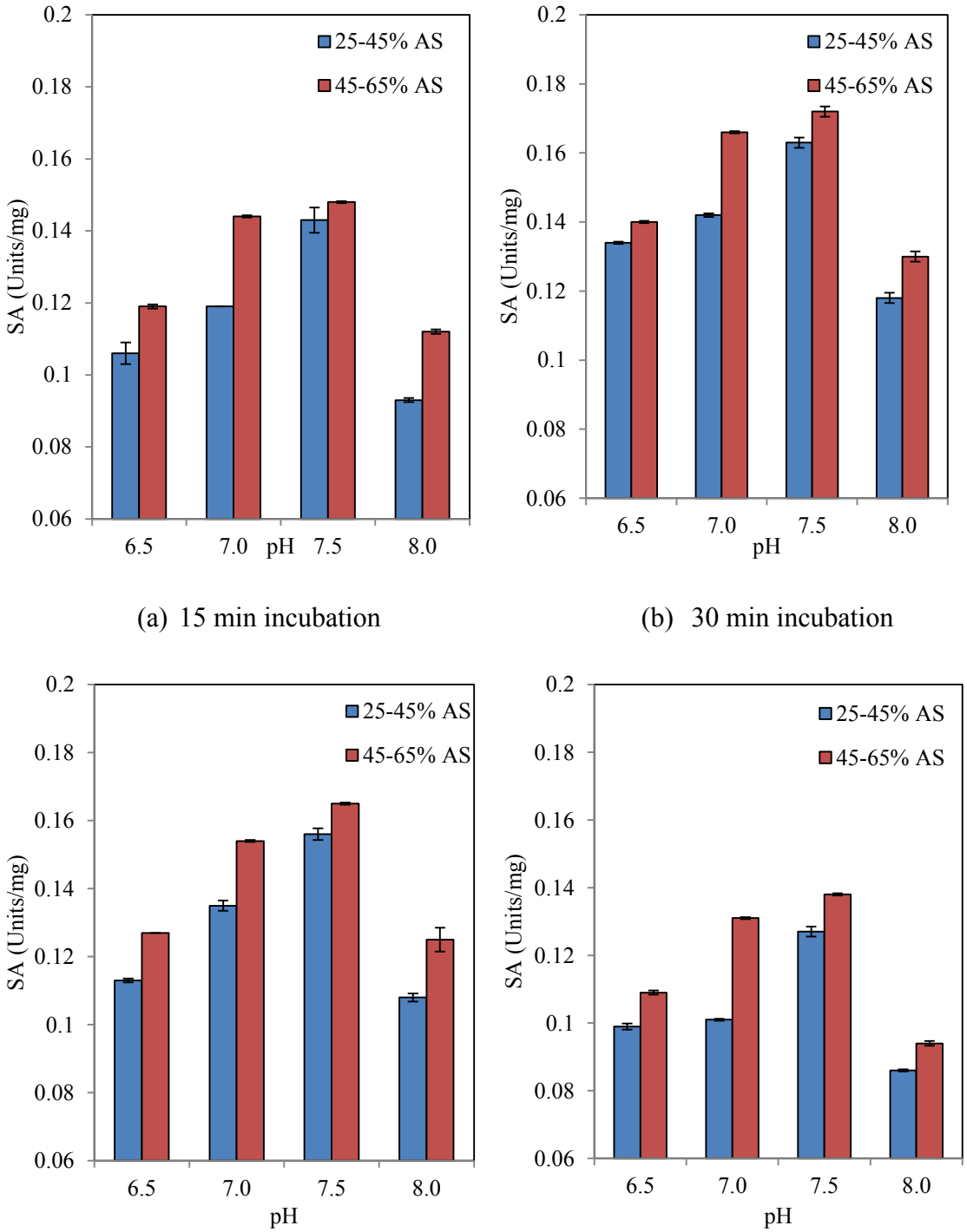
Figure 6.36. Effect of incubation time on the specific activity (SA) of the cystatin extracted from the bovine lung and pancreas mixture at different pH and ammonium sulphate saturation levels (mean ± std, n=3).

0.163 to 0.127 Units/mg (22%) and from 0.118 to 0.086 Units/mg (27.1%) at the pH levels of 6.5, 7.0, 7.5 and 8.0, respectively. Similar trends were observed with the SA of cystatin extracted from the bovine pancreas and the mixture of lungs and pancreas samples. All the three samples (bovine lung, pancreas and mixture of lungs and pancreas samples) also exhibited similar trends with the 45-65% ammonium sulphate saturation at all pH levels. The highest SA of cystatin was observed at the incubation time of 30 min for all samples at all pH levels.

6.2.3.4. Effect of Ammonium Sulphate Saturation on Specific Activity (SA): The effect of ammonium sulphate saturation (25-45% and 45-65%) on the specific activity (SA) of the cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different pH levels (6.5, 7.0, 7.5, and 8.0) and incubation times (15, 30, 45 and 60 min) are shown in Figures 6.37 - 6.39. With the 15 min incubation, increasing the ammonium sulphate saturation from 25-45% to 45-65% increased the SA of the cystatin from the bovine lung sample from 0.106 to 0.119 Units/mg (12.2%), from 0.119 to 0.144 Units/mg (21%), from 0.143 to 0.148 Units/mg (3.4%) and from 0.093 to 0.112 Units/mg (20.4%) for the pH levels of 6.5, 7.0, 7.5 and 8.0, respectively. Similar trends were observed for the SA of the cystatin extracted from the three samples (bovine lung sample, pancreas sample and mixture of lungs and pancreas sample) at the incubation times of 30, 45 and 60 min. Increasing the ammonium sulphate saturation level from 25-45% to 45-65% increased the SA of cystatin.

6.2.4. Yield (Y)

The effects of sample types (bovine lung, pancreas and mixture of lung and pancreas), pH (6.5, 7.0, 7.5, and 8.0), incubation time (15, 30, 45 and 60 min) and ammonium sulphate saturation level (25-45% and 45-65%) on the yield (Y) of cystatin are shown in Tables 6.12 - 6.14. The yield (Y) of cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas varied from 24.08 to 17.22%, from 19.22 to 10.91% and 21.59 to 16.18%, respectively.



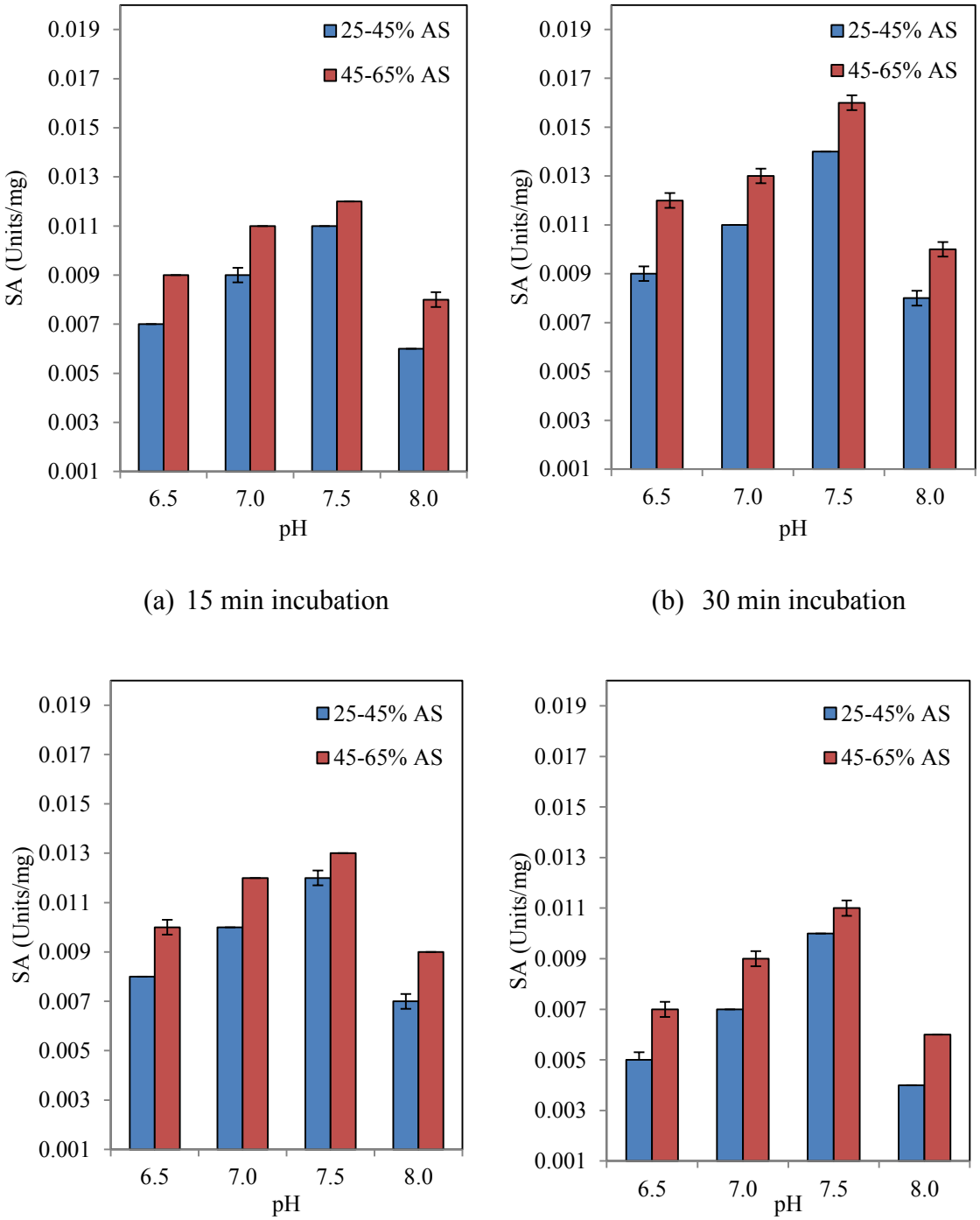
(a) 15 min incubation

(b) 30 min incubation

(c) 45 min incubation

(d) 60 min incubation

Figure 6.37. Effect of ammonium sulphate (AS) saturation on the specific activity (SA) of the cystatin extracted from the bovine lung at different pH levels and incubation times (mean ± std, n=3).



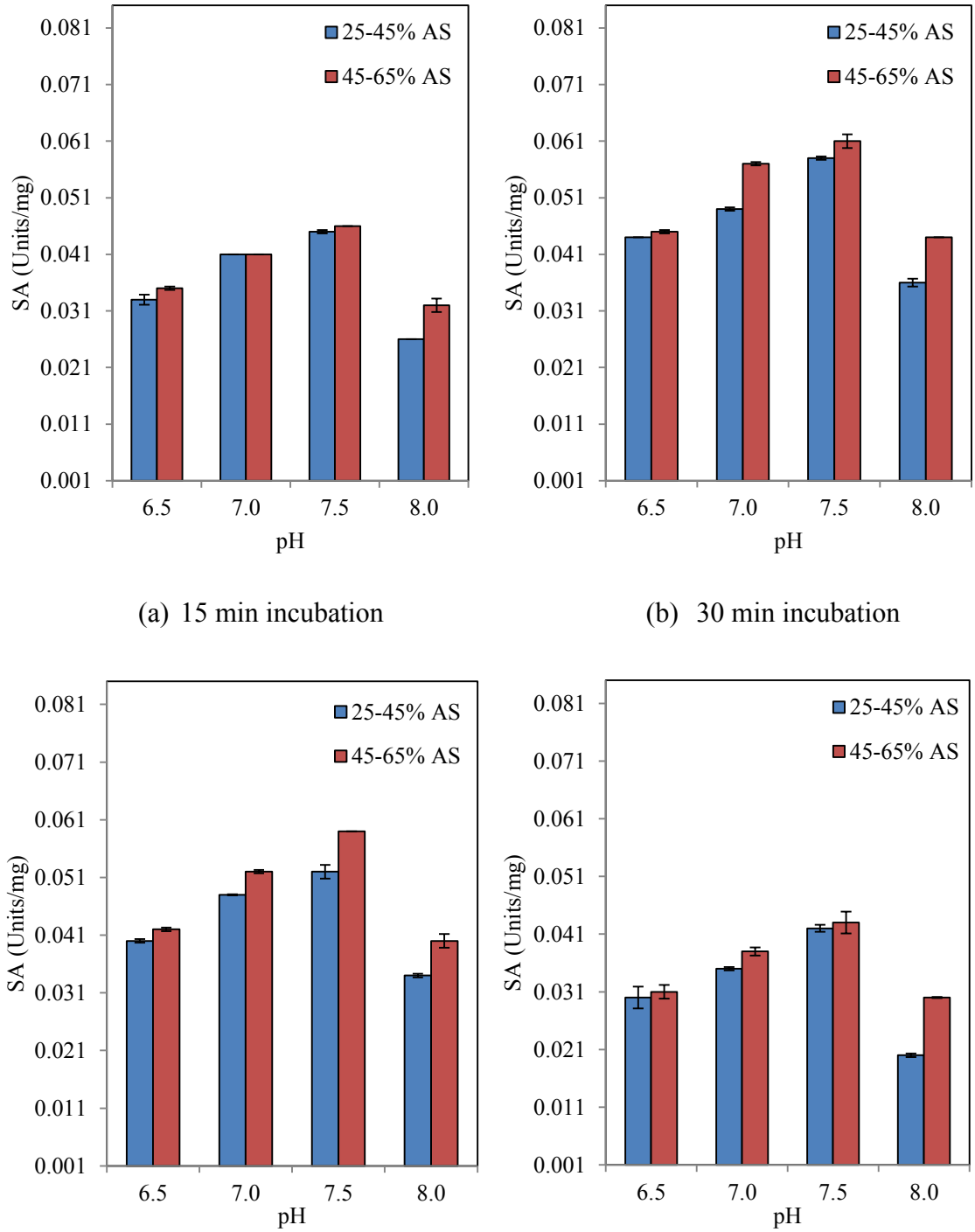
(a) 15 min incubation

(b) 30 min incubation

(c) 45 min incubation

(d) 60 min incubation

Figure 6.38. Effect of ammonium sulphate (AS) saturation on specific activity (SA) of the cystatin extracted from the bovine pancreas at different pH levels and incubation times (mean ± std, n=3).



(a) 15 min incubation

(b) 30 min incubation

(c) 45 min incubation

(d) 60 min incubation

Figure 6.39. Effect of ammonium sulphate (AS) saturation on the specific activity (SA) of the cystatin extracted from the bovine lung and pancreas mixture at different pH levels and incubation times (mean ± std, n=3).

Analysis of variance was performed on the yield data using Minitab (Version 16.2.3, Minitab Inc, State College, Pennsylvania, USA). The results are shown in Table 6.21. The effects of sample type, pH level, incubation time and ammonium sulphate saturation level were significant at the 0.001 level. The two, three and four way interactions between the parameters were also significant at the 0.001 level.

The Tukey grouping was also performed on the yield (Y) data and shown in Table 6.22. The bovine lung, pancreas and mixture of lung and pancreas samples were significantly different from each other at the 0.05 level. The highest average yield (Y) of cystatin of 29.58% was obtained from the bovine lung sample. All incubation times were significantly different from each other at the 0.05 level. The highest average yield (Y) of cystatin of 28.53% was obtained at 30 min incubation time. All pH levels were significantly different from each other at the 0.05 level. The highest average yield (Y) of cystatin of 32.30% was obtained at pH 7.5. The two ammonium sulphate saturation levels were also significantly different from each other at the 0.05 level. The highest average yield (Y) of cystatin of 25.53% was obtained with 45-65% ammonium sulphate saturation.

6.2.4.1. Effect of Sample Type on Yield (Y): The effects of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on yield (Y) of the cystatin extracted at different incubation times (15, 30, 45 and 60min) and saturation levels (25-45% and 45-65%) are shown in Figures 6.38 - 6.41. With the 25-45 % saturation, pH 6.5 and 15 min incubation, the yield (Y) of the cystatin extracted from the bovine lung was 24.08%, which was higher than those extracted from the pancreas and mixture of lung and pancreas samples. All three samples (bovine lung, pancreas and mixture of lung and pancreas) exhibited similar trends with the 45-65% ammonium sulphate saturation, pH levels (7.0, 7.5 and 8.0) and incubation times (30, 45 and 60 min). The bovine lung sample showed higher yield (Y) of the cystatin than the pancreas and mixture of lung and pancreas samples under all extraction conditions.

Table 6.21. Analysis of variance for yield (Y) - ammonium sulphate extraction

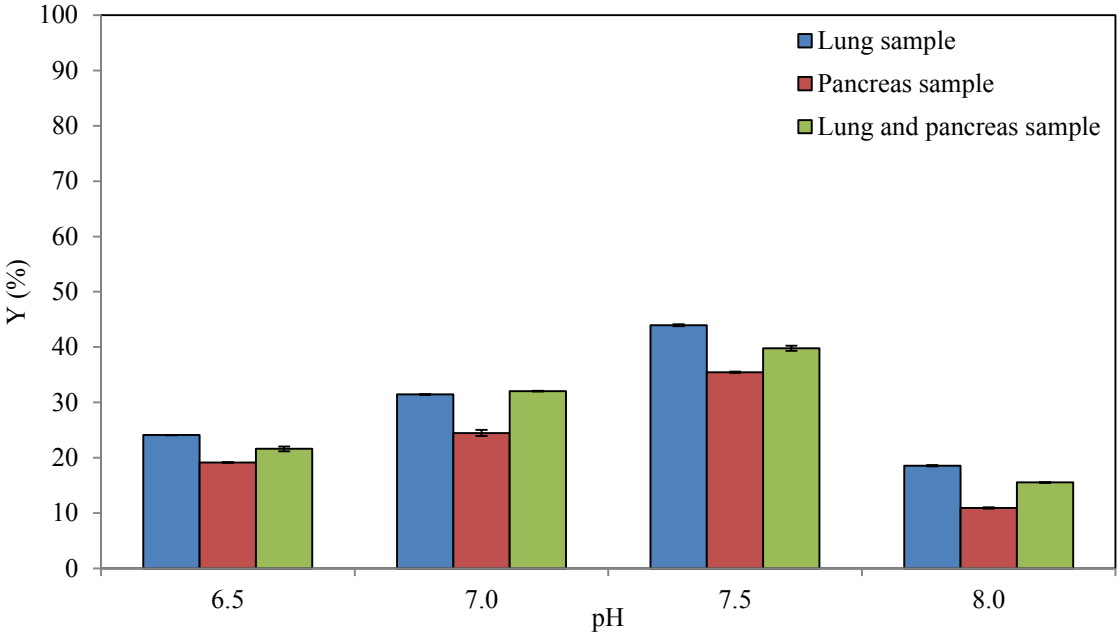
Source	DF	SS	MS	F	P
Total	287	26204.5			
Model					
ST	2	6843.5	3421.77	8964.94	0.001
IT	3	4646.2	1548.74	4057.65	0.001
PH	3	11358.2	3786.06	9919.38	0.001
SL	1	1150.7	1150.68	3014.75	0.001
ST*IT	6	240.0	39.99	104.78	0.001
ST*PH	6	189.2	31.53	82.59	0.001
ST*SL	2	59.6	29.82	78.12	0.001
IT*PH	9	1097.9	121.99	319.62	0.001
IT*SL	3	85.5	28.50	74.68	0.001
PH*SL	3	58.0	19.34	50.67	0.001
ST*IT*PH	18	235.4	13.08	34.27	0.001
ST*IT*SL	6	65.5	10.92	28.61	0.001
IT*PH*SL	9	45.3	5.03	13.18	0.001
ST*IT*PH*SL	18	53.9	2.99	7.84	0.001
Error	198	75.6	0.38		

ST : Sample type
IT : Incubation time (min)
PH : pH
SL : Saturation level (%)
DF : Degree of freedom
SS : Sum of square
MS : Mean of square
R^2 : 0.9977
CV : 5.4%

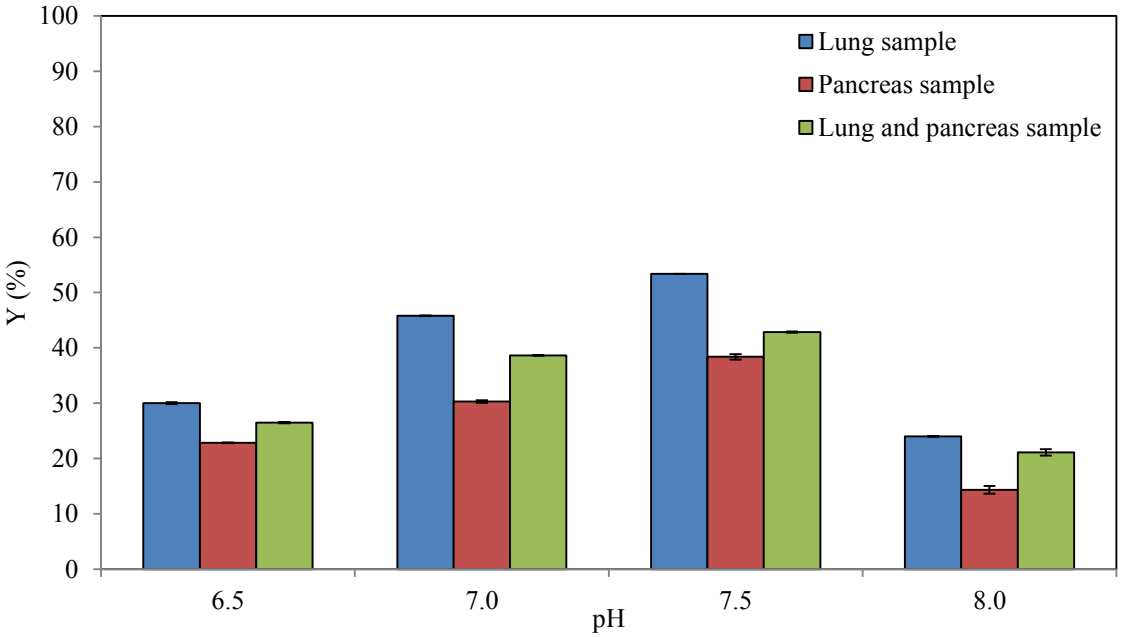
Table 6.22. Tukey grouping for yield (Y) - ammonium sulphate extraction

Factors	Level	N	Mean yield (%)	Tukey Grouping
Sample Type	Lung sample	96	29.58	A
	Lung and pancreas sample	96	23.39	B
	Pancreas sample	96	17.62	C
Incubation Time (min)	30	72	28.53	A
	15	72	25.05	B
	45	72	23.11	C
	60	72	17.42	D
pH	7.5	72	32.30	A
	7.0	72	26.18	B
	6.5	72	19.91	C
	6.0	72	15.73	D
Saturation Level (%)	45-65%	144	25.53	A
	25-45%	144	21.52	B

Means that share the same letter are not significantly different from one another at the 005 level.

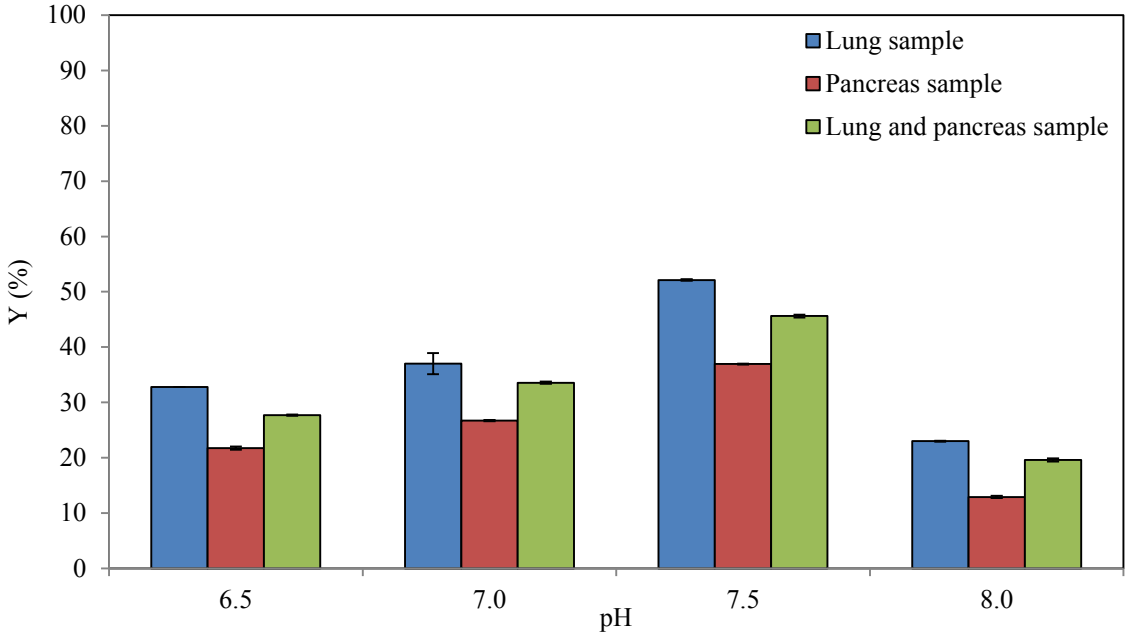


(a) 25-45% ammonium sulphate saturation

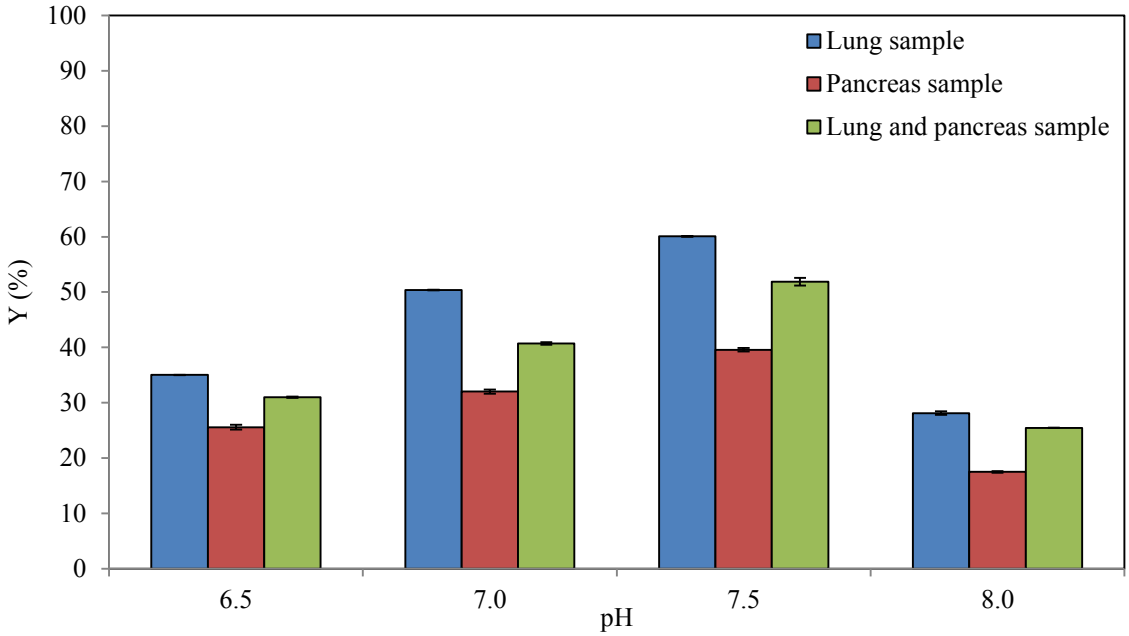


(b) 45-65% ammonium sulphate saturation

Figure 6.38. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on yield (Y) of the cystatin extracted at 15 min incubation and different levels of pH and ammonium sulphate saturation

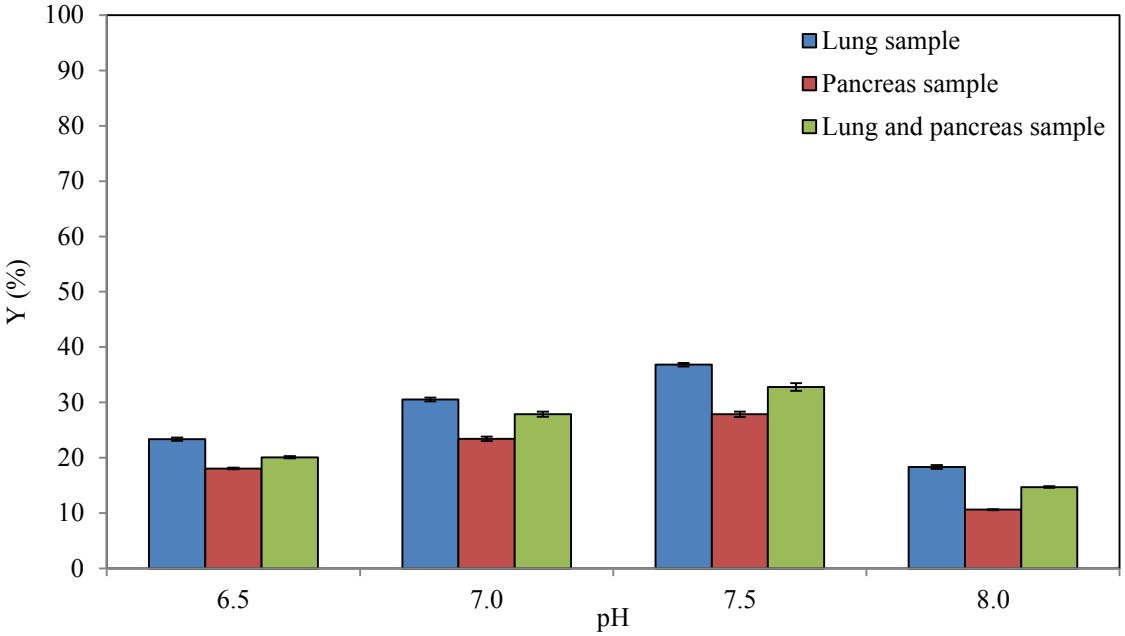


(a) 25-45% ammonium sulphate saturation

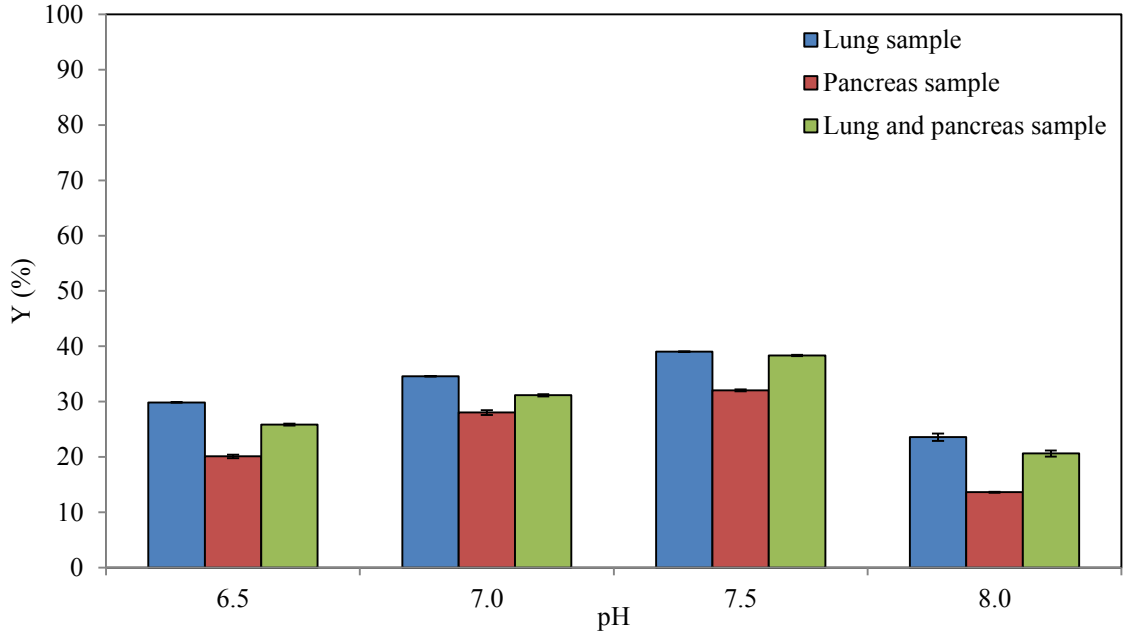


(b) 45-65% ammonium sulphate saturation

Figure 6.39. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on yield (Y) of the cystatin extracted at 30 min incubation and different levels of pH and ammonium sulphate saturation

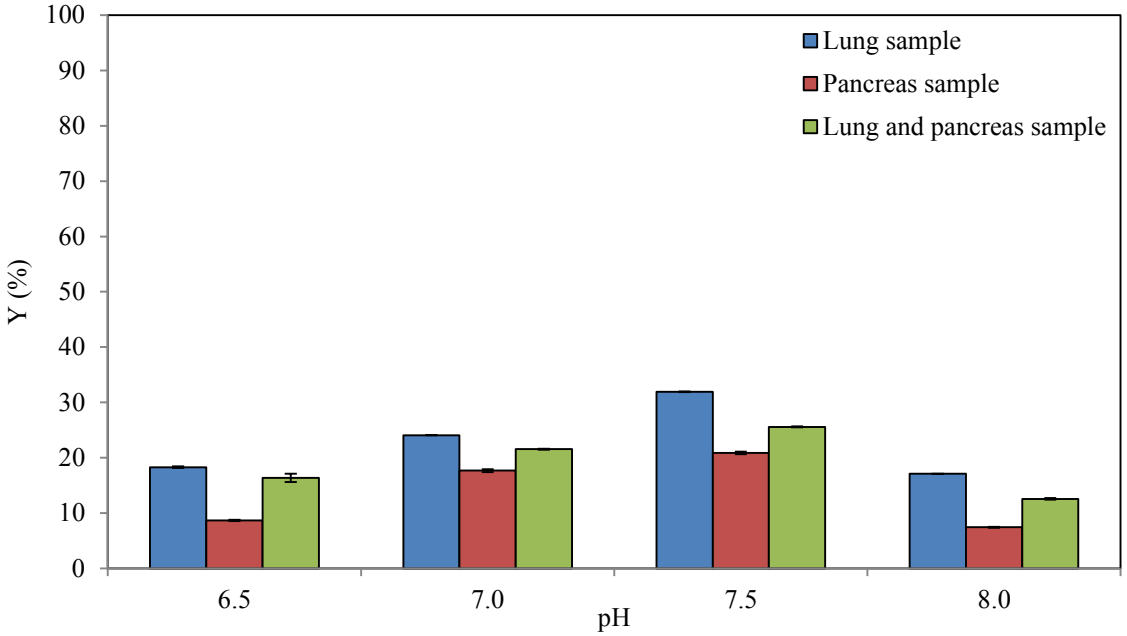


(a) 25-45% ammonium sulphate saturation

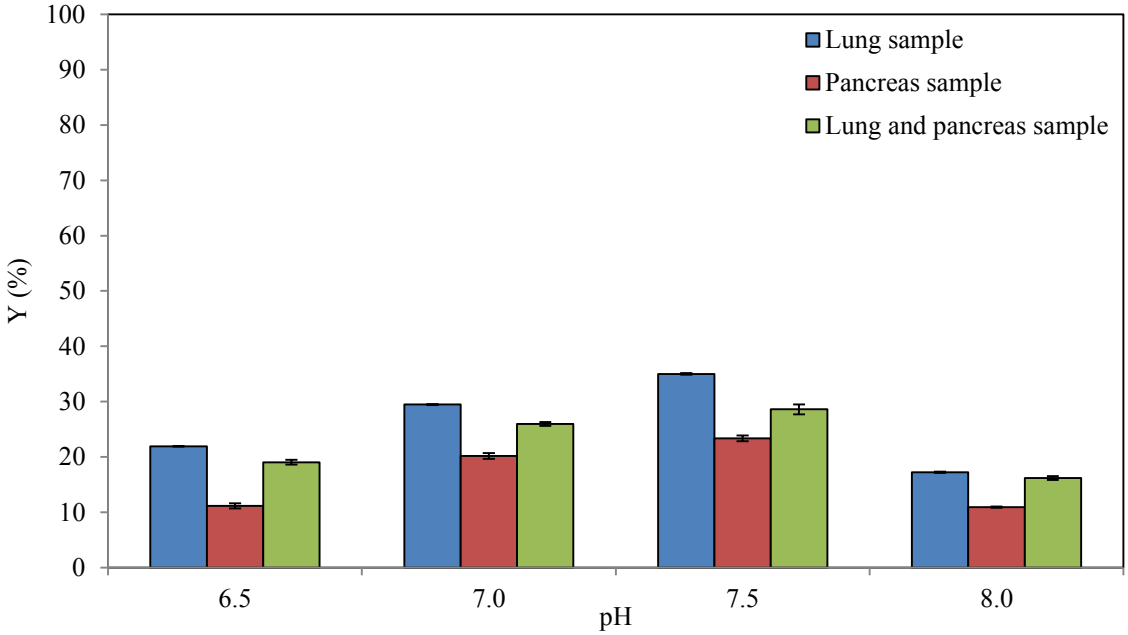


(b) 45-65% ammonium sulphate saturation

Figure 6.40. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on yield (Y) of the cystatin extracted at 45 min incubation and different levels of pH and ammonium sulphate saturation



(a) 25-45% ammonium sulphate saturation

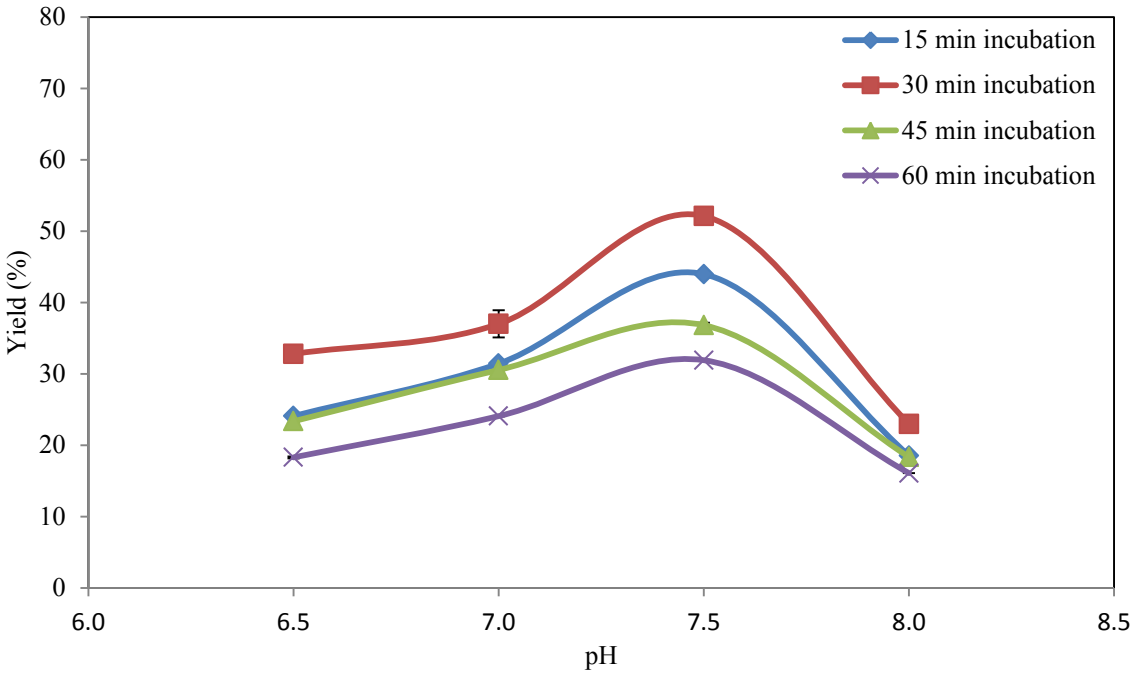


(b) 45-65% ammonium sulphate saturation

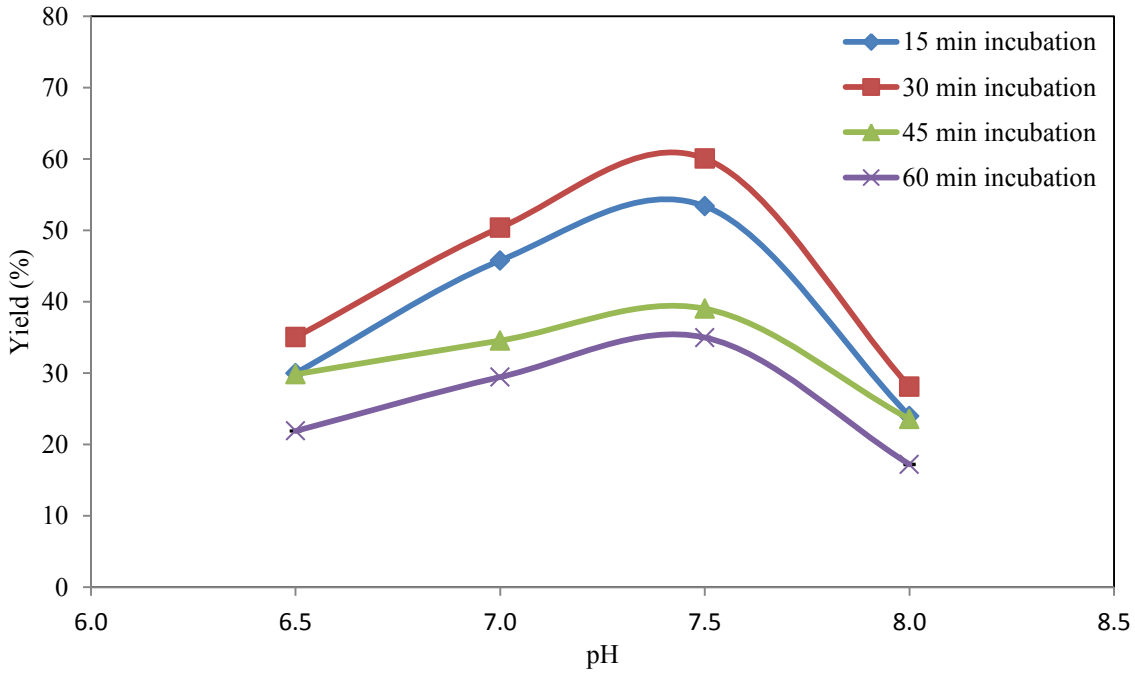
Figure 6.41. Effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples) on yield (Y) of the cystatin extracted at 60 min incubation and different levels of pH and ammonium sulphate saturation

6.2.4.2. Effect of pH on Yield (Y): The effect of pH (6.5, 7.0, 7.5, and 8.0) on the yield (Y) of the cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different incubation times (15, 30, 45 and 60min) and saturation levels (25-45% 45-65%) are shown in Figures 6.42 - 6.44. The SD for the graphs are too small (<2). With the 25-45% saturation ammonium sulphate saturation, increasing the pH from 6.5 to 7.5 increased the Y of the cystatin from the bovine lung sample from 24.08 to 43.97% (82.6%), from 32.79 to 52.12% (58.9%), from 23.35 to 36.82% (57.68%) and from 18.29 to 31.91% (74.4%) for the incubation times of 15, 30, 45 and 60 min, respectively. A further increase in the pH (from 7.5to 8.0) decreased the Y of the cystatin from 43.97 to 18.54% (57.83%), from 52.12 to 22.98% (55.9%), from 36.82 to 18.3% (50.2%) and from 31.91 to 18.3% (42.6%) at all incubation times respectively. Similar trends were observed for the bovine pancreas and mixture of lungs and pancreas samples. All the three samples (bovine lung, pancreas and mixture of lungs and pancreas samples) also exhibited similar trends with the 45-65% ammonium sulphate saturation. The highest cystatin yield was produced at the pH 7.5 under all extraction conditions.

6.2.4.3. Effect of incubation on Yield (Y): The effect of incubation time (15, 30, 45 and 60 min) on the yield (Y) of the cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different pH (6.5, 7.0, 7.5, and 8.0) and ammonium sulphate saturation levels (25-45% and 45-65%) are shown in Figures 6.45 - 6.47. The SD for the graphs are too small (<2). With the 25-45% ammonium sulphate saturation, increasing the incubation time from 15 to 30 min increased the Y of the cystatin extracted from the bovine lung sample from 24.08 to 32.79% (36.1%), from 31.43 to 36.99% (17.6%), from 43.97 to 52.12% (18.5%) and from 18.54 to 22.98% (23.9%) at the pH levels of 6.5, 7.0, 7.5 and 8.0 respectively. A further increase in the incubation time from 30 to 60 min decreased the Y of cystatin from 32.79 to 18.29% (44.2%), 36.99 to 24.07% (34.9%), 52.12 to 31.91% (57.9%), and 22.98 to 16.08% (30%) at pH levels 6.5, 7.0, 7.5 and 8.0, respectively. Similar trends were observed for the Y of cystatin extracted from the bovine pancreas and mixture of lungs and pancreas samples. All the three samples (bovine lung, pancreas and mixture of lungs and pancreas samples) also exhibited similar trends with the 45-65% ammonium sulphate saturation. The highest Y of cystatin was observed at the incubation.

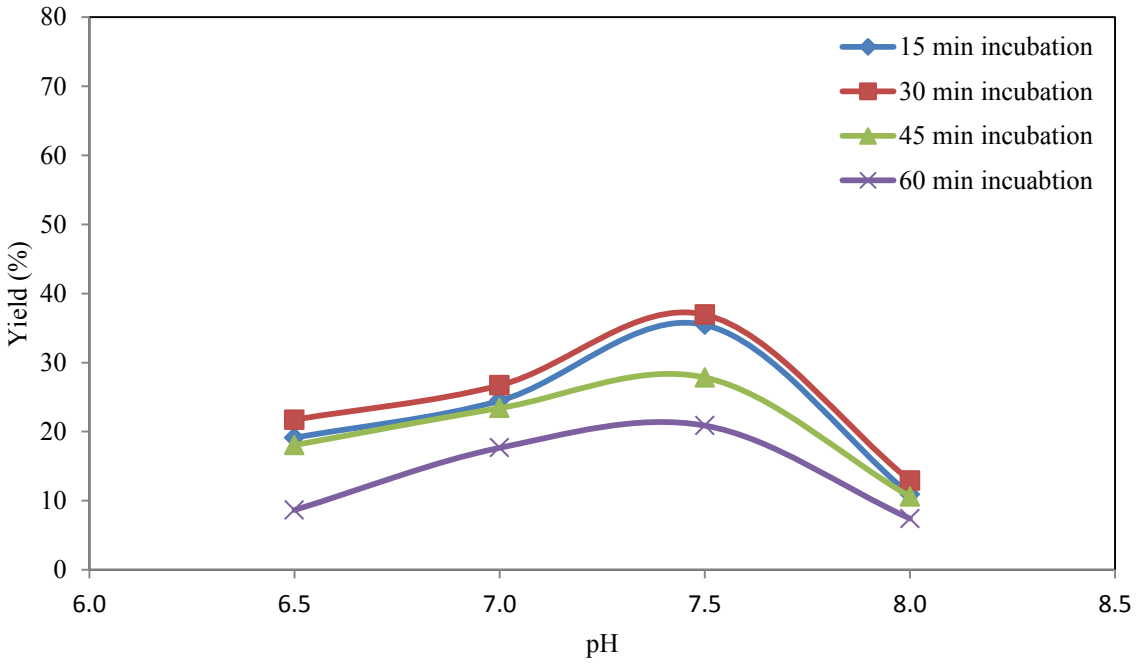


(b) 25-45% ammonium sulphate saturation

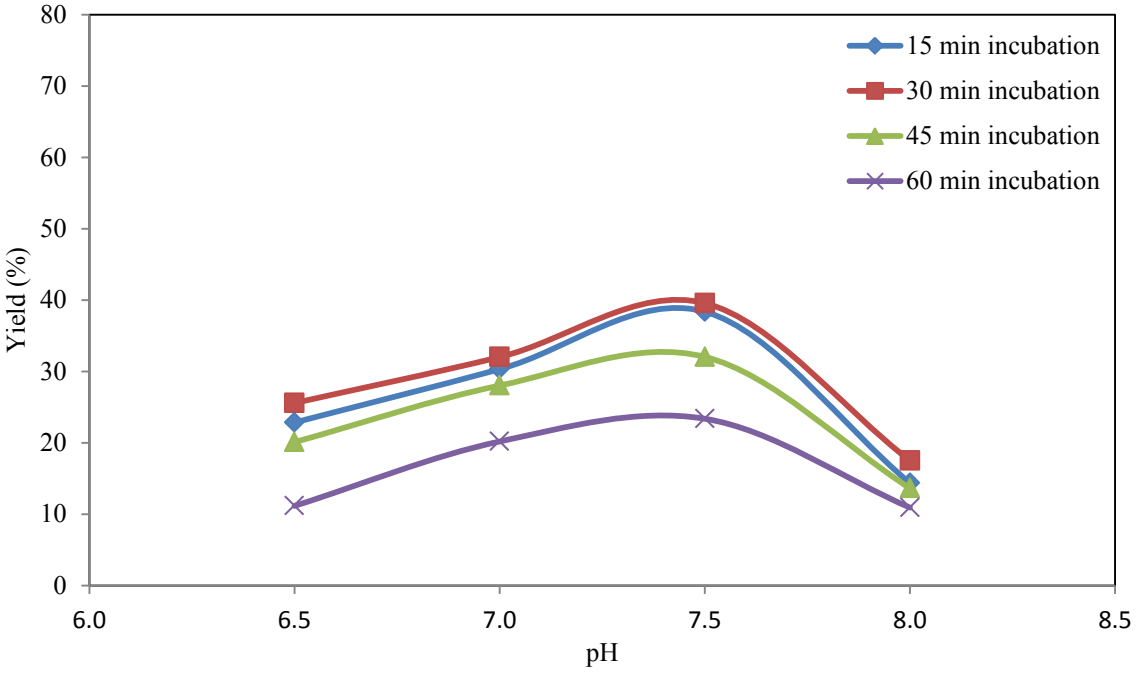


(b) 45-65% ammonium sulphate saturation

Figure 6.42. Effect of pH on the yield (Y) of the cystatin extracted from the bovine lung at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3)

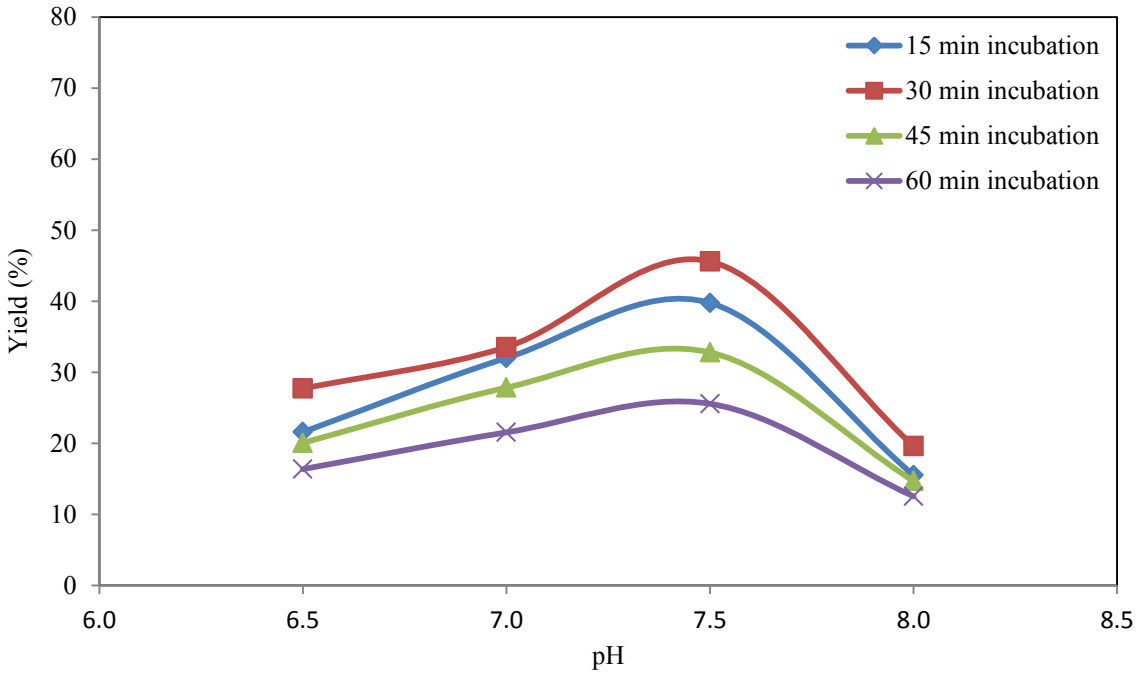


(a) 25-45% ammonium sulphate saturation

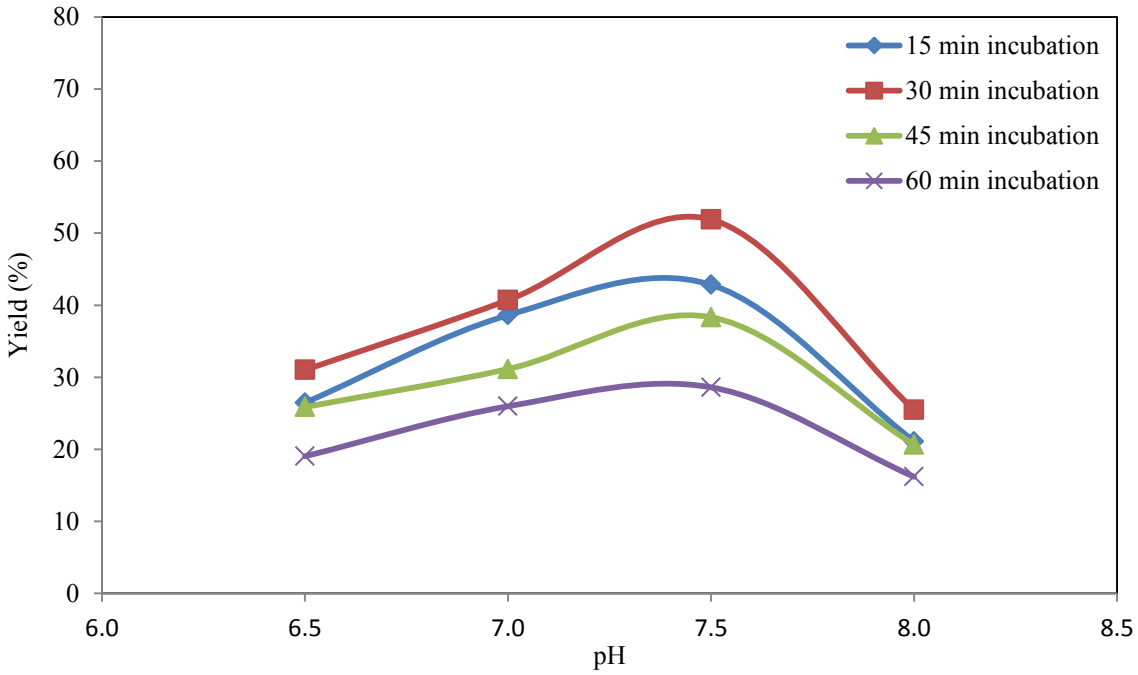


(b) 45-65% ammonium sulphate saturation

Figure 6.43. Effect of pH on the yield (Y) of the cystatin extracted from the bovine pancreas at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3)

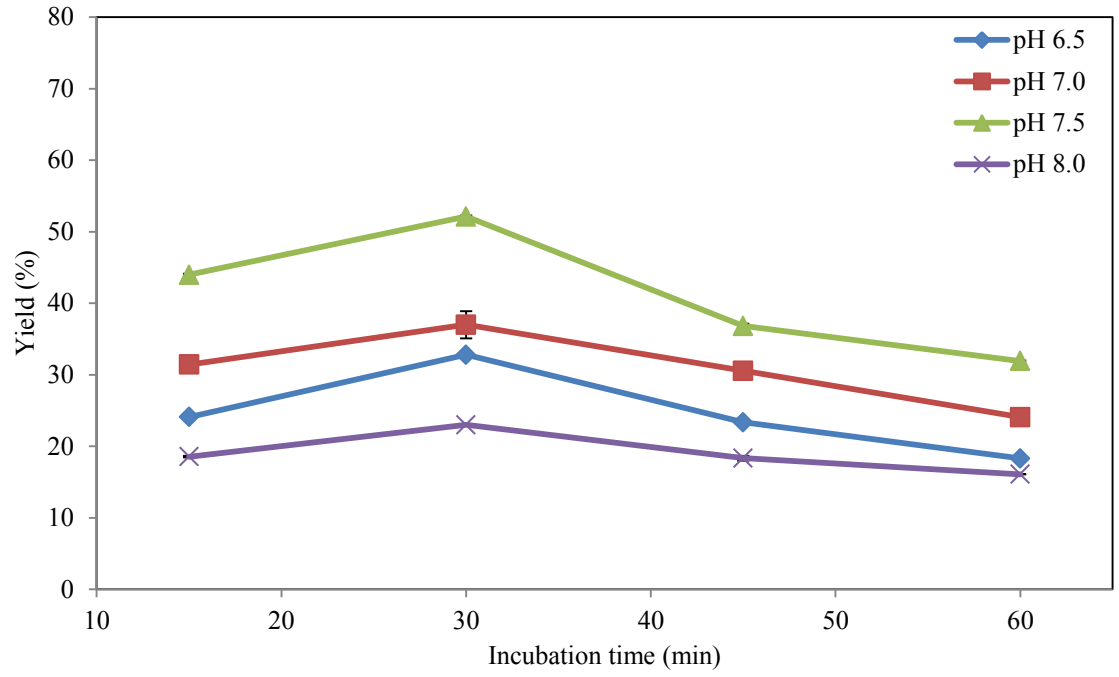


(a) 25-45% ammonium sulphate saturation

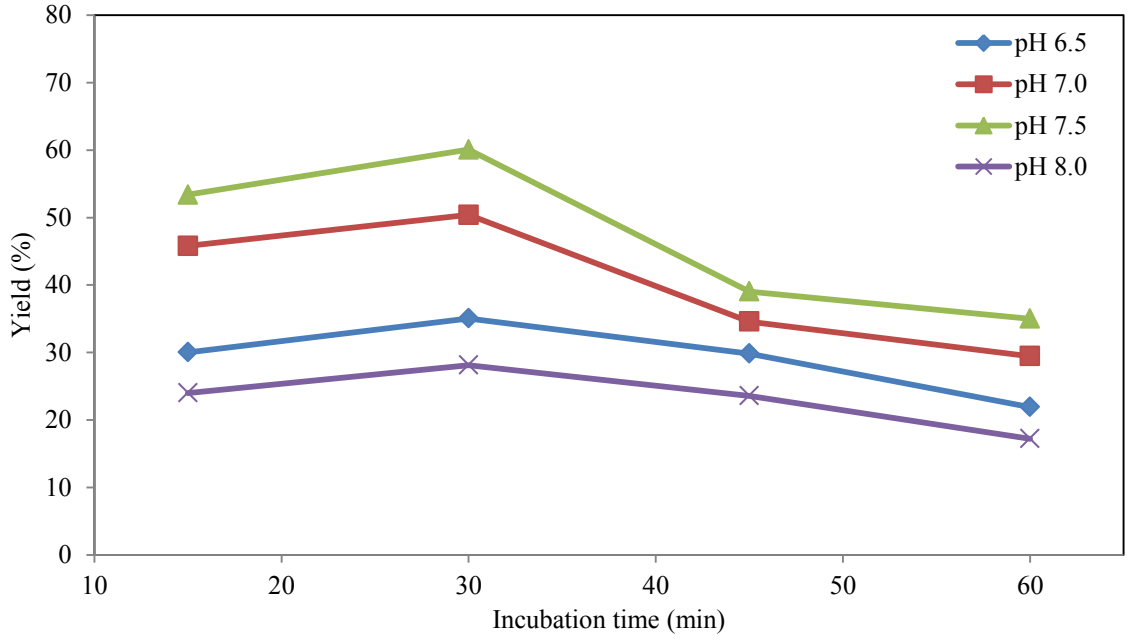


(b) 45-65% ammonium sulphate saturation

Figure 6.44. Effect of pH on the yield (Y) of cystatin extracted from the bovine lung and pancreas mixture at different incubation times and ammonium sulphate saturation levels (mean ± std, n=3)

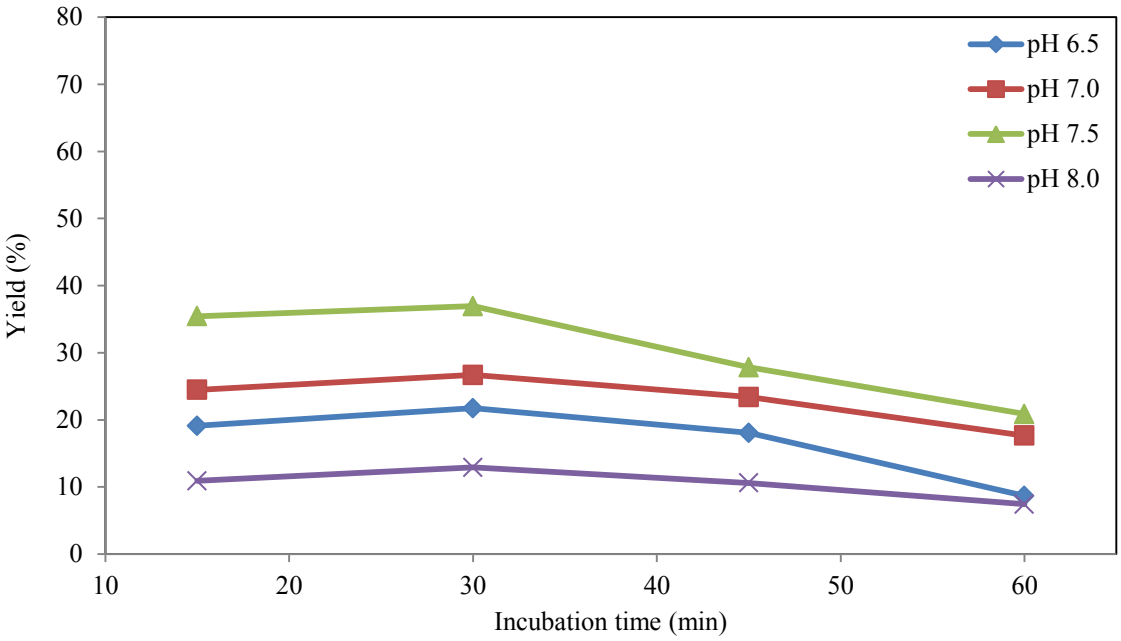


(a) 25-45% ammonium sulphate saturation

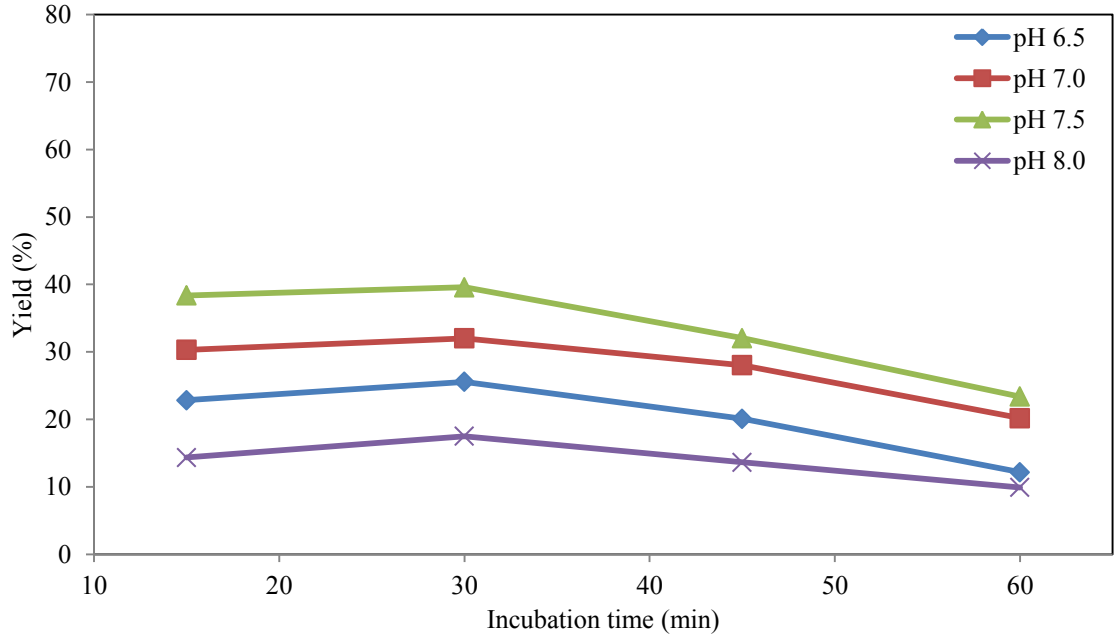


(b) 45-65% ammonium sulphate saturation

Figure 6.45. Effect of incubation on the yield (Y) of the cystatin extracted from the bovine lung at different levels of pH and ammonium sulphate saturation levels (mean ± std, n=3)

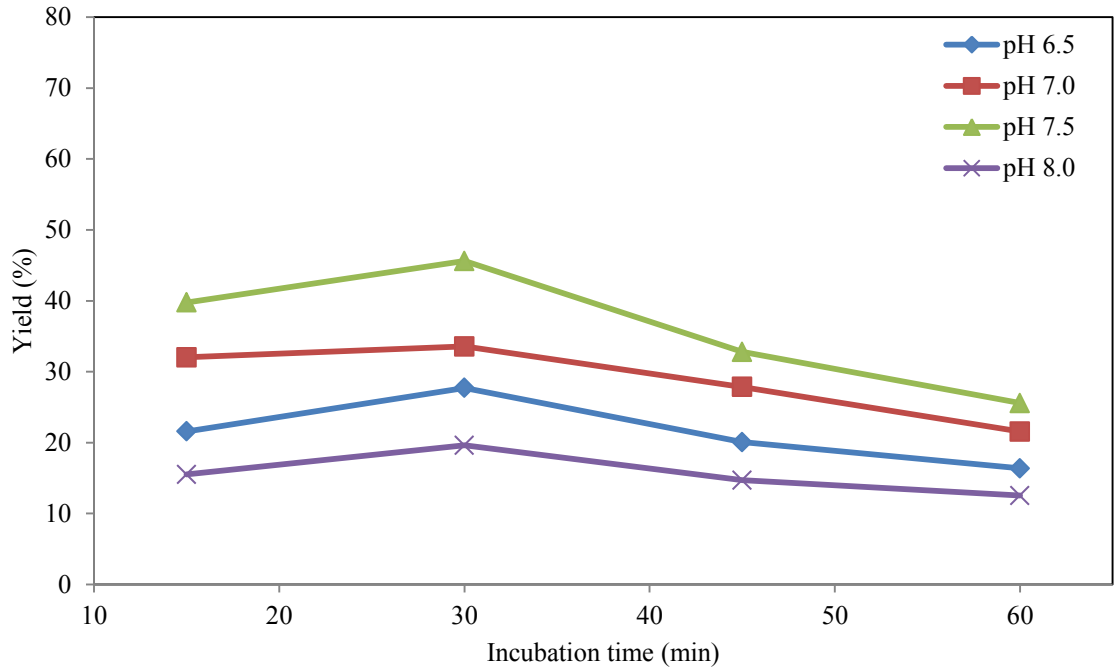


(a) 25-45% ammonium sulphate saturation

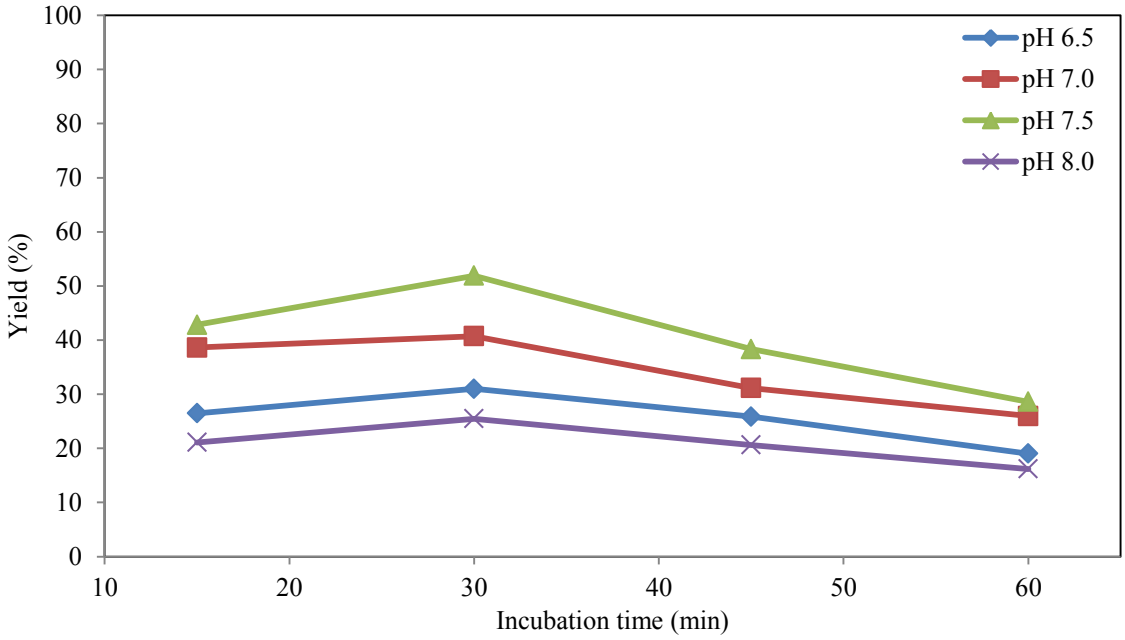


(b) 45-65% ammonium sulphate saturation

Figure 6.46. Effect of incubation on the yield (Y) of the cystatin extracted from the bovine pancreas at different levels of pH and ammonium sulphate saturation levels (mean ± std, n=3)



(a) 25-45% ammonium sulphate saturation



(b) 45-65% ammonium sulphate saturation

Figure 6.47. Effect of incubation on the yield (Y) of the cystatin extracted from the bovine lung and pancreas mixture at different levels of pH and ammonium sulphate saturation levels (mean ± std, n=3)

time of 30 min for all samples at all pH levels.

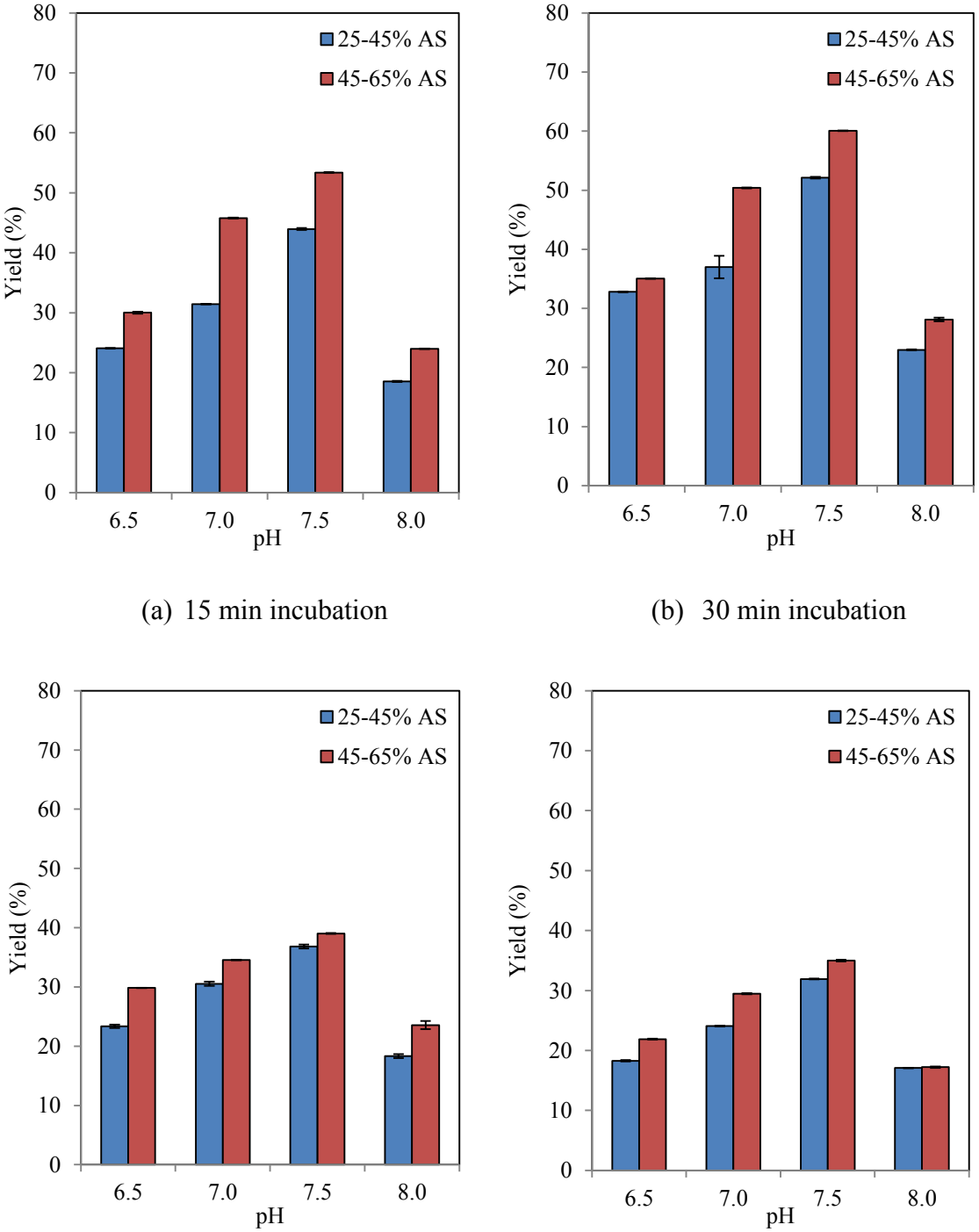
6.2.4.4. Effect of ammonium sulphate saturation on Yield (Y): The effect of ammonium sulphate saturation (25-45% and 45-65%) on the yield (Y) of cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples at different pH levels (6.5, 7.0, 7.5, and 8.0) and incubation times (15, 30, 45 and 60 min) are shown in Figures 6.48 - 6.50. With the 15 min incubation, increasing the ammonium sulphate saturation from 25-45% to 45-65% increased the Y of the cystatin extracted from bovine lung sample from 24.08 to 30.01% (24.6%), from 31.43 to 45.79% (45.6%), from 43.97 to 53.39% (21.4%) and from 18.54 to 24.00% (29.7%) at the pH levels of 6.5, 7.0, 7.5 and 8.0, respectively. Similar trends were observed for the Y of the cystatin extracted from the three samples (bovine lung sample, pancreas sample and mixture of lungs and pancreas sample) at other incubation times (30, 45 and 60min). Increasing the ammonium sulphate saturation level (from 25-45% to 45-65%) increased the Y of cystatin.

6.3. Effect of pH and Temperature in Inhibitory Assay

The effects of pH (2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5 and 11.5) and temperature (30, 40, 50, 60, 70, 80 and 90 ºC) the inhibitory activity of the bovine lung, pancreas, and mixture of lung and pancreas samples with 45-65% ammonium sulphate saturation, pH 7.5 and incubation time of 30 mins are shown in Tables 6.24 and 6.25 respectively.

6.3.1. Effect of pH in Inhibitory Assay

The effect of pH (2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5 and 11.5) on the maximal inhibitory activity of cystatin was evaluated at 37ºC. The results are shown in Figure 6.51.The bovine lung, pancreas, and mixture of lung and pancreas samples showed maximal inhibitory activity of 55.1%, 23.8 % and 39.1% at pH 7.5, respectively. pH 2 and 11 showed zero inhibitory activity for all the three samples (bovine lung sample, pancreas sample, and mixture of lung and pancreas sample). The bovine Lung recorded the maximum inhibitory activity



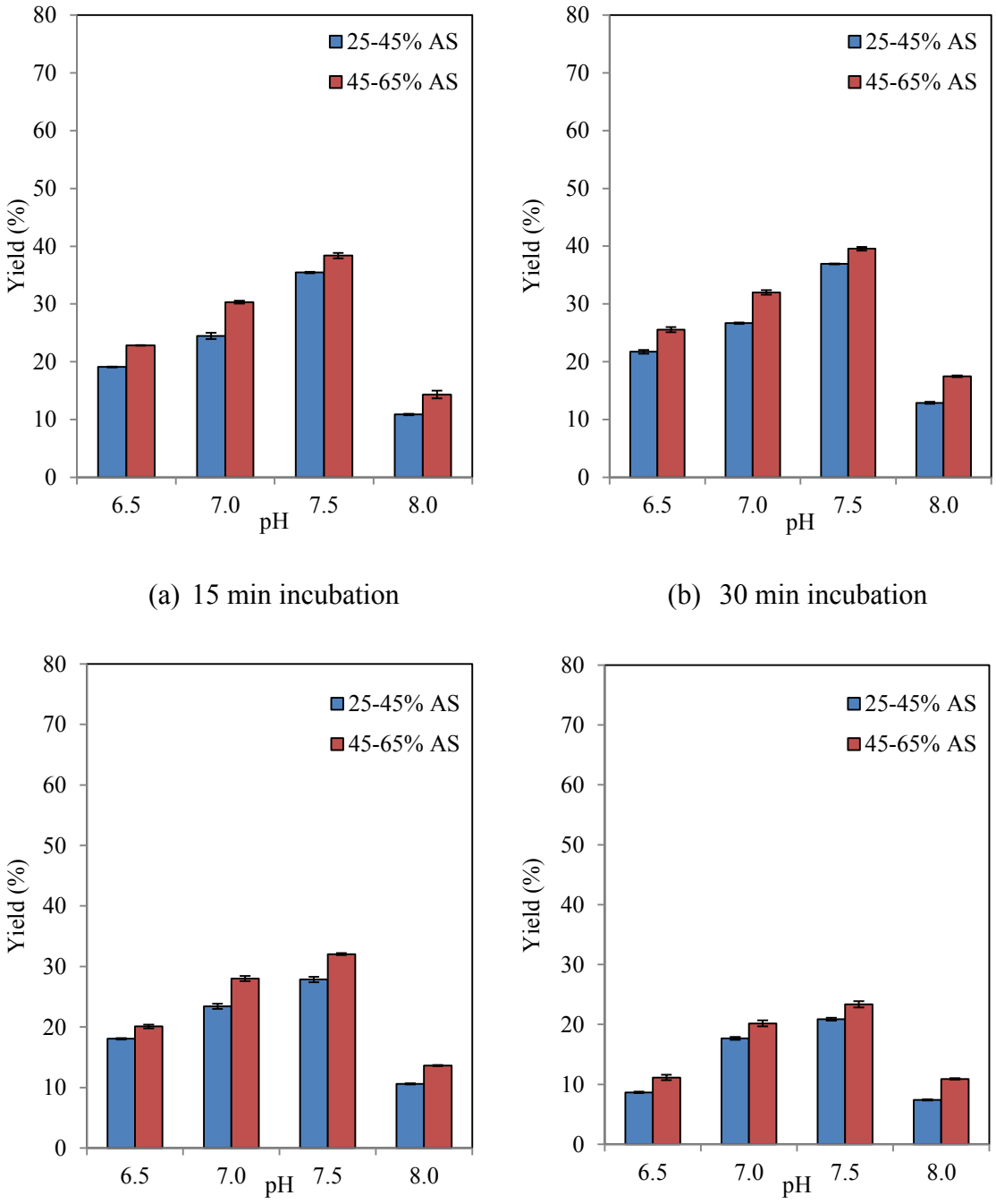
(a) 15 min incubation

(b) 30 min incubation

(c) 45 min incubation

(d) 60 min incubation

Figure 6.48. Effect of ammonium sulphate (AS) saturation on the yield (Y) of the cystatin extracted from the bovine lung at different incubation times and pH levels (mean ± std, n=3)



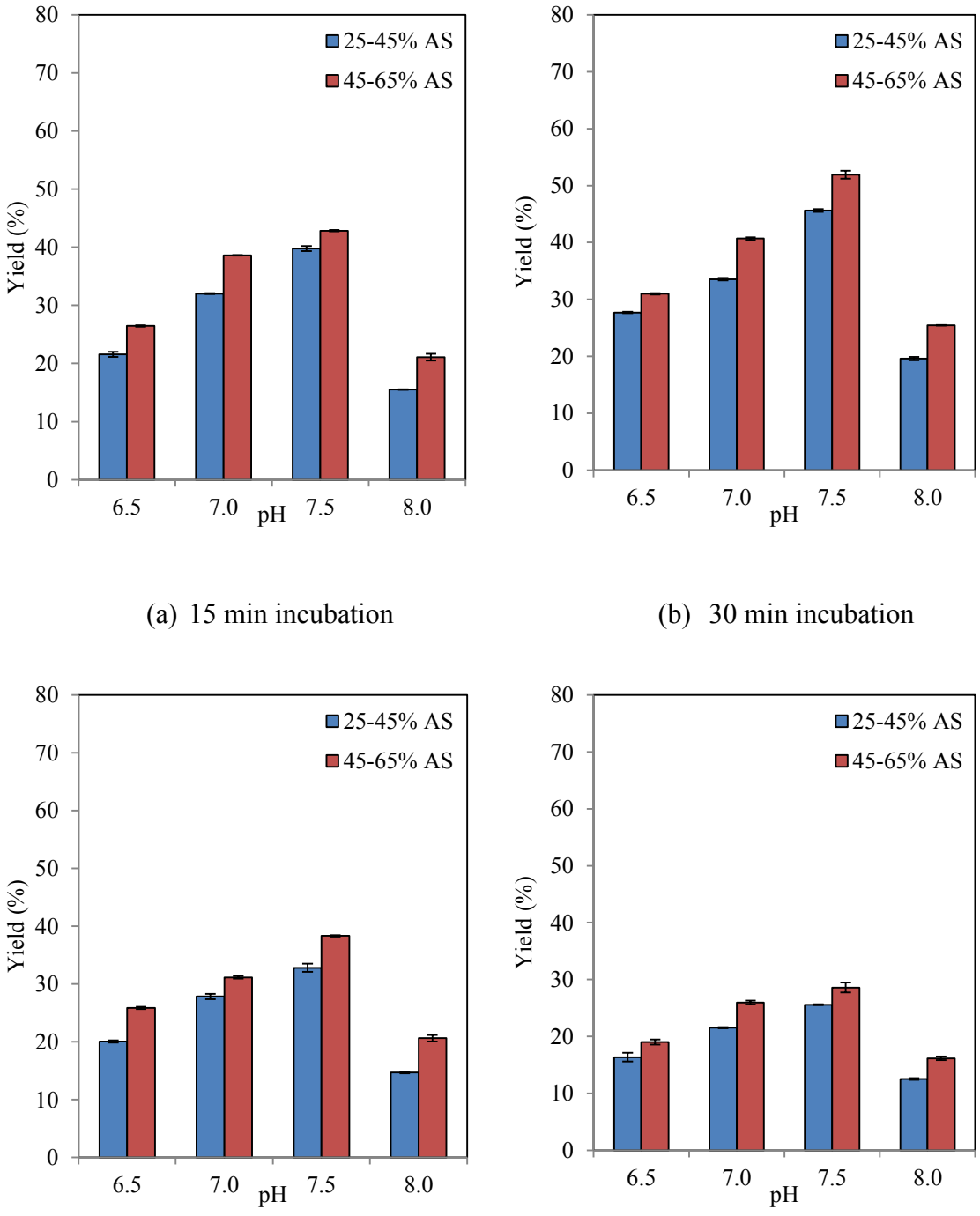
(a) 15 min incubation

(b) 30 min incubation

(c) 45 min incubation

(d) 60 min incubation

Figure 6.49. Effect ammonium sulphate (AS) saturation on the yield (Y) of the cystatin extracted from the bovine pancreas at different incubation times and pH levels (mean ± std, n=3)



(a) 15 min incubation

(b) 30 min incubation

(c) 45 min incubation

(d) 60 min incubation

Figure 6.50. Effect of ammonium sulphate (AS) saturation on the yield (Y) of the cystatin extracted from the bovine lung and pancreas mixture at different incubation times and pH levels (mean ± std, n=3)

Table 6.24. Effect of pH on inhibitory activity of cystatin from lung, pancreas and mixture of lung and pancreas (extracted at 45%-65% ammonium sulphate saturation, 30 min incubation time and pH-7.5)

pH	Inhibition (%) of cystatin		
	Lung sample	Pancreas sample	Lung and pancreas sample
2.5	0.0±0.00	0.0±0.00	0.0±0.00
3.5	1.9±0.05	0.8±0.12	1.2±0.37
4.5	11.5±0.30	7.4±0.47	9.1±0.26
5.5	17.9±0.52	10.0±1.11	13.2±0.74
6.5	48.8±0.90	19.4±1.48	29.3±1.15
7.5	55.1±1.59	23.8±1.14	39.7±0.77
8.5	44.4±0.15	15.2±2.25	31.2±0.55
9.5	38.6±0.30	12.4±1.85	23.1±1.86
10.5	29.7±0.89	9.6±1.45	19.1±1.26
11.5	0.0±0.00	0.0±0.00	0.0±0.00

Replicates (n) =3

Table 6.25. Effect of temperature on inhibitory activity of cystatin from lung, pancreas and mixture of lung and pancreas samples (extracted at 45%-65% ammonium sulphate saturation, 30min incubation time and pH-7.5)

Temperature	Inhibition (%) of cystatin		
	Lung sample	Pancreas sample	Lung and pancreas sample
30	42.5±1.14	12.9±0.77	37.4±0.73
40	55.2±0.75	19.5±0.17	40.1±1.02
50	56.8±0.52	25.8±0.37	43.8±0.20
60	44.6±1.06	21.4±0.55	38.6±0.60
70	38.5±0.64	16.7±0.25	31.4±0.45
80	35.4±1.02	11.1±0.77	27.2±0.46
90	28.3±0.81	9.4±0.62	19.6±0.26

Replicates (n) =3

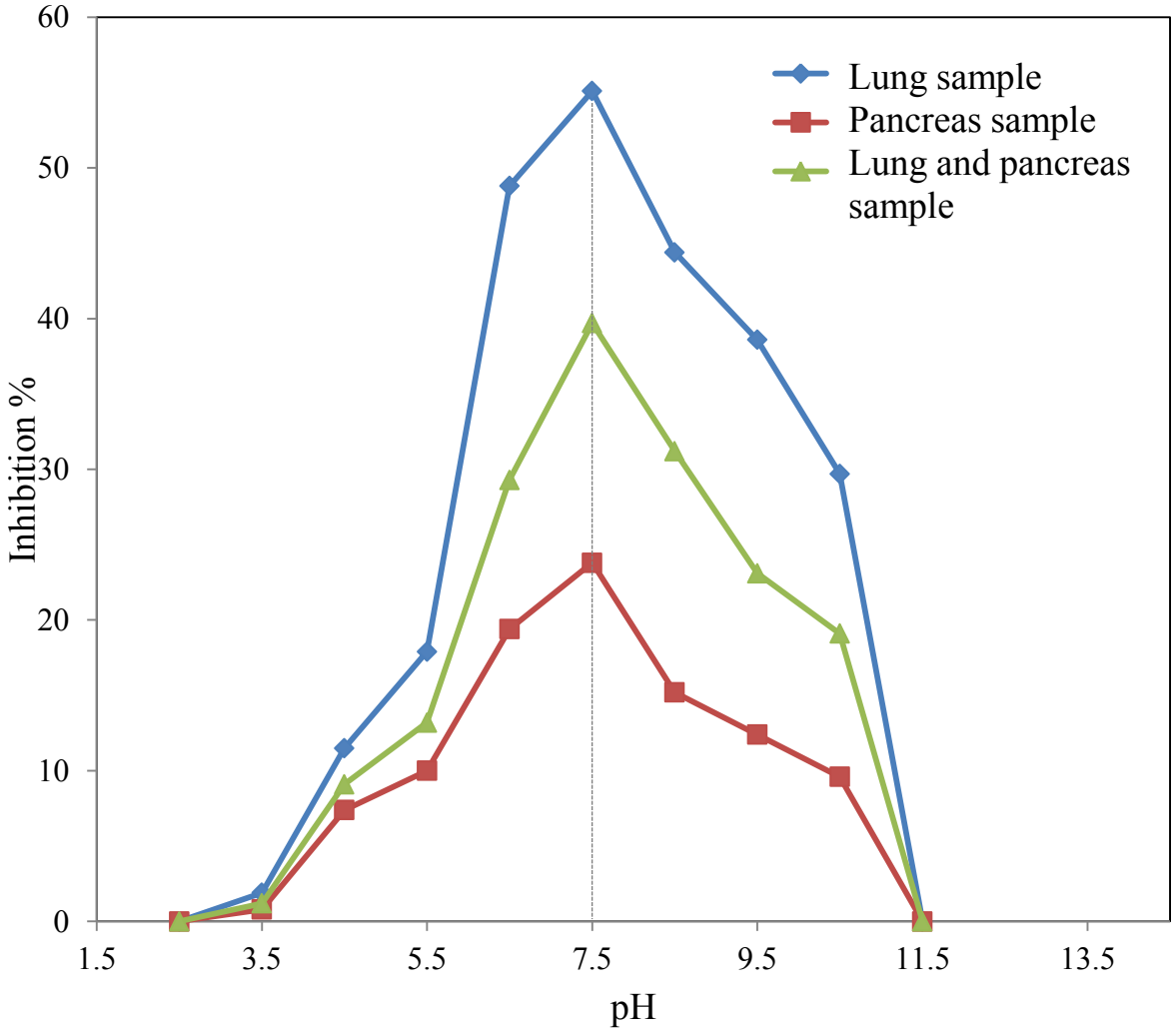


Figure 6.51. Effect of pH on inhibition activity of the cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples (parameters- 30 min of incubation time, pH 7.5, 45-65% ammonium sulphate saturation)

6.3.2. Effect of temperature (T ºC) in Inhibitory Assay

The effect of temperature (30, 40, 50, 60, 70, 80 and 90 ºC) on the maximal inhibitory activity of cystatin was evaluated at the pH 7.5. The results are shown Figure 6.52. The bovine lung sample, pancreas sample, and mixture of lung and pancreas sample showed maximal inhibitory activity of 56.8%, 25.8% and 43.8% at 50 ºC, respectively. Bovine lung recorded the maximum inhibitory activity of cystatin.

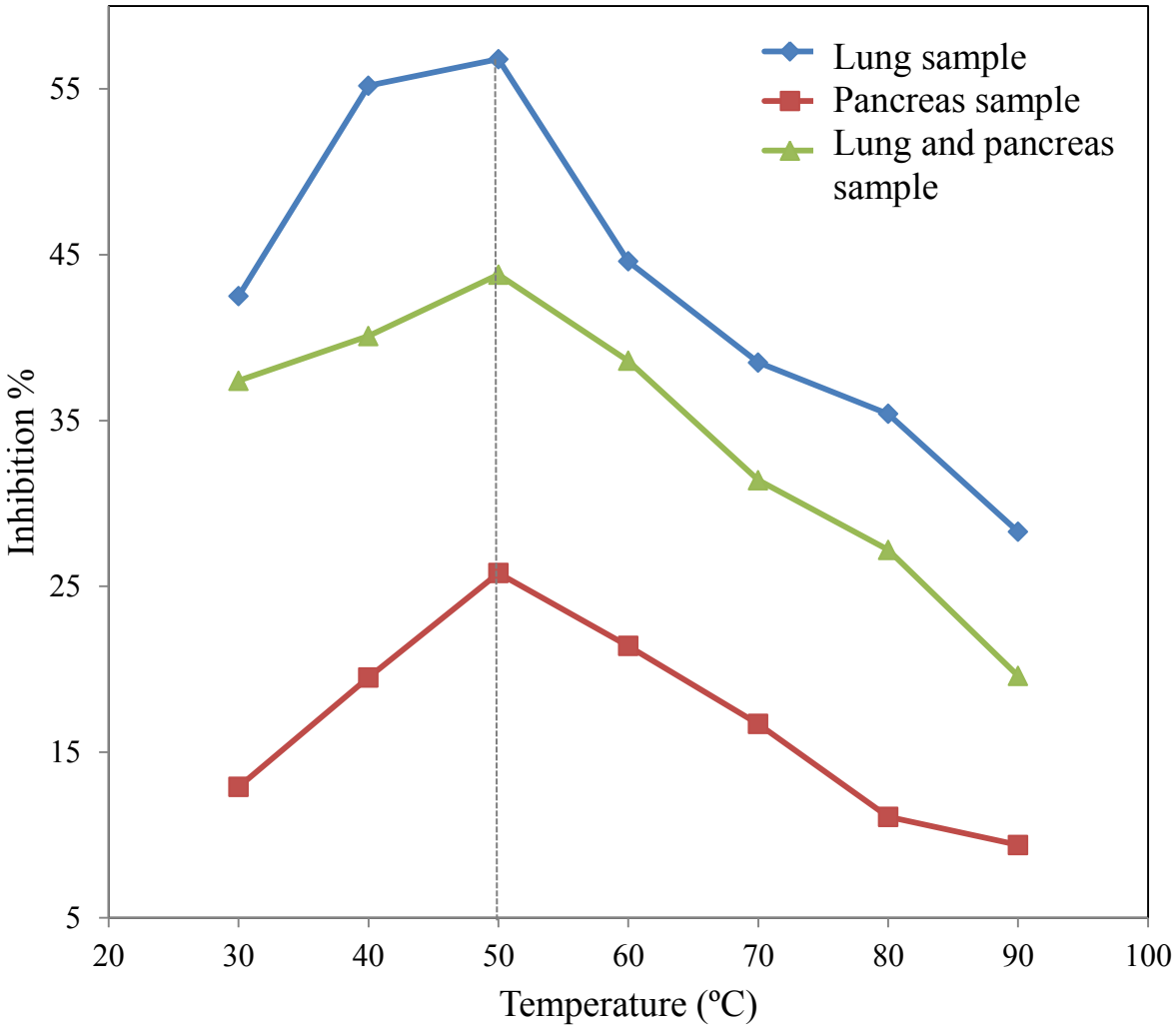


Figure 6.52. Effect of temperature on inhibition activity of the cystatin extracted from the bovine lung, pancreas and mixture of lung and pancreas samples (parameters- 30 min of incubation time, pH 7.5, 45-65% ammonium sulphate saturation).

CHAPTER 7. DISCUSSION

7.1. Crude Extraction

In the present study, homogenization of all samples (bovine lung, pancreas and mixture of lung and pancreas samples) was first carried out and the volume (V), total protein (TP), total activity (TA), specific activity (SA) and yield (Y) were recorded. The yield (Y) was set to 100%. The alkali treatment was then applied to the crude extract. The optimum pH of 11.0 was used for digestions of tissue in the homogenate which caused less degradation of proteins/peptides present in the sample (Somerville, 2002). After centrifugation and alkaline treatment of the homogenized samples, there were decreases observed in the volume (V), total protein (TP), total activity (TA) and yield (Y) of the cystatin extracted from the three samples. However, the specific activity (SA) of cystatin was increased.

The alkaline treatment facilitated the digestion of the homogenate by degradation of protein, thereby decreasing the total protein (TP) and total activity (TA) and yield (Y) of cystatin. Warwas and Sawicki (1988) reported that the major advantage of the alkaline treatment was the deactivation of endogenous proteases present in the sample which resulted in the efficient extraction of the cystatin. Murphy et al. (2009) suggested that alkaline treatment should be considered by rendering and packing industries as it helps in the production of sterilized product.

The yield (Y) of cystatin obtained after alkaline treatment was 90.30% (9.23%), 79.90% (19.73%) and 82.70% (17.21%) for the bovine lung, pancreas and mixture of lung and pancreas samples. The bovine lung sample has higher yield of cystatin than the bovine pancreas and mixture of lung and pancreas samples because of the high specificity for inhibition papain (cysteine protease) (Khan and Bano, 2008).

After alkaline treatment, the bovine lung sample showed a TP of 7569 mg, a TA of 760.9 Units, an SA of 0.100 Units/mg, a Y of 90.7%. The bovine pancreas sample showed a TP of 10242 mg, a TA of 62.3 Units, an SA of 0.006 Units/mg, a Y of 79.0%. The mixture of bovine lung and pancreas sample showed a TP of 13835 mg, a TA of

246.6 Units, an SA of 0.018 Units/mg, a Y of 82.7%. The results obtained from this study are comparable to those reported in the literature. Khan and Bano (2008) extracted cystatin from goat lungs and reported a TP of 4950 mg, a TA of 1019.7 Units, a SA of 0.206 Units/mg and Y of 79.7%. Sadaf et al (2005) applied the alkaline treatment on the crude extraction to extract cystatin from goat kidney and reported a TP of 1344 mg, a TA of 14.4 Units, an SA of 0.01 Units/mg and a Y of 97%. Priyadarshini and Bano (2009) extracted cystatin from goat pancreas and reported TP of 2899 mg, TA of 76 Units, SA of 0.026 Units/mg and Y of 71.6%.

7.2. Ammonium Sulphate Extraction

During ammonium sulphate extraction the proteins and nucleic acids are purified under optimal conditions of pH, solvents, and ammonium sulfate saturation level (Atkinson et al., 1983; Foster, 1994). The incubation time is also considered to be an important parameter as it allows protein denaturation (Khan and Bano, 2008). In the present study, the effect of sample type (bovine lung, pancreas and mixture of lung and pancreas samples), incubation time (15, 30, 45, 60 mins), pH (6.5, 7.0, 7.5, 8.0) and ammonium sulphate saturation level (25-45% and 45-65%) on TP (total protein), TA (total activity), SA (specific activity) and Y (yield) were investigated in order to determine the optimal conditions for the extraction of cystatins by ammonium sulphate extraction method. The experiment was carried out at a temperature of $4^{O}C$. Dialysis was performed against the buffer, before assay in order to remove and dissolve the formed precipitate as recommended by Nuhayati et al. (2013).

7.2.1. Effect of Sample Type

In the present study, the ammonium sulphate extraction method was used for extracting cystatin from the bovine lung, pancreas and a mixture of lung and pancreas. The highest total protein (TP), total activity (TA) and yield (Y) of the cystatin were obtained from the bovine lung sample at 30 min incubation, pH 7.5 and 45-65% ammonium sulphate saturation. Sumbul and Bano (2006) reported that samples with high

specificity of inhibition towards papain will provide higher total activity, specific activity and yield of cystatin.

7.2.1.1. Effect of Sample Type on Total Protein (TP): The total protein (TP) of the cystatin obtained with ammonium sulphate extraction at a 30 min incubation time, a pH of 7.5 and an ammonium sulphate saturation of 45-65% was 2788.7 mg, 2214.7 mg and 2526.0 mg for the bovine lung, pancreas and mixture of lung and pancreas samples, respectively. The bovine lung sample recorded the highest total protein (TP) than the bovine pancreas and the mixture of lung and pancreas samples.

The results obtained from the study are comparable to those reported in the literature. Khan and Bano (2008) extracted the cystatin from goat lung with ammonium sulphate and reported a total protein (TP) of 3375 mg. Priyadarshini and Bano (2009) extracted of cystatin from goat pancreas and reported a total protein (TP) of 147.7 mg due to acetone fractionation . Tsushima et al. (1995) extracted cystatin from bovine hoof and reported a TP of 14.0 mg.

7.2.1.2. Effect of Sample Type on Total Activity (TA): The total activity (TA) of the cystatin observed with ammonium sulphate extraction at a 30 min incubation time, a pH of 7.5 and 45-65% ammonium sulphate saturation was 505.8 Units, 29.8 Units and 154.6 Units for the bovine lung, pancreas and mixture of lung and pancreas samples, respectively. The bovine lung sample recorded higher total activity (TA) due to the high specificity towards inhibition of cysteine protease activity in papain.

Similar results have been reported by other authors. A total activity (TA) of 725.6 Units was reported by Khan and Bano (2008) for cystatin extracted from goat lung with ammonium sulphate. Priyadarshini and Bano (2009) reported a total activity (TA) of 63.39 Units for cystatin extracted from goat pancreas with ammonium sulphate. Baba et al. (2005) reported a TA of 85.3 Units for cystatin extracted from sheep plasma. Kirihara et al. (1995) reported a TP of 339 Units for cystatin extracted from bovine colostrum. These studies showed that goat lung sample provided high total activity (TA) than other samples.

7.2.1.3. Effect of Sample Type on Specific Activity (SA): The specific activity (SA) of the cystatin obtained with ammonium sulphate extraction at a 30 min incubation time, a pH of 7.5 and an ammonium sulphate saturation of 45-65% was 0.172 Units/mg, 0.016 Units/mg and 0.061 Units/mg for the bovine lung, pancreas and mixture of lung and pancreas samples, respectively. The bovine lung sample recorded highest specific activity (SA) because it had inhibition greater specificity towards cysteine proteases than for the serine, threonine, aspartic, glutamic and metalloproteases (Sumbul and Bano, 2006).

The results obtained from this study are comparable to those reported in the literature. The specific activity (SA) reported for the cystatin extracted from the goat kidney and goat pancreas were 0.215 Units/mg and 0.371 Units/mg (Khan and Bano, 2008; Priyadarshini and Bano, 2009). Baba et al. (1995) reported a SA of 0.909 Units/mg for cystatin extracted from sheep plasma. Lee et al. (2008) reported a SA of 0.880 Units/mg for cystatin extracted from pig plasma.

7.2.1.4. Effect of Sample Type on Yield (Y): The yield (Y) of the cystatin obtained by ammonium sulphate extraction at a 30 min incubation time, a pH of 7.5 and an ammonium sulphate saturation of 45-65% was 60.07%, 39.57% and 51.89% for the bovine lung, pancreas and mixture of lung and pancreas samples, respectively. The bovine lung sample recorded highest yield (Y) because of its high inhibitory activity and specificity towards papain (Khan and Bano, 2008).

The results obtained from the present study are comparable to those reported in the literature. Khan and Bano (2008) obtained a cystatin yield of 68% from goat kidney with ammonium sulphate extraction. Priyadarshini and Bano (2009) produced a cystatin yield of 51.6% from goat pancreas with ammonium sulphate. Nuhayati et al. (2013) reported a yield of 17.4% for cystatin extracted from catfish.

7.2.2. Effect of Incubation Time

During the extraction process, incubation time is considered to be an important parameter as it favors the yield and purity of proteinase inhibitor. Unwanted and impure proteins are degraded in the incubation process (Sheabar et al., 2003). In the present

study, the effect of incubation time (increasing the incubation time from 15 mins through 60 mins) on the TP (total protein), TA (total activity), specific activity (SA) and yield (Y) of cystatin was variant for both ammonium sulphate saturations (25-45% and 45-65%) at pH levels (6.5, 7.0, 7.5 and 8.0) for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples).

7.2.2.1. Effect of Incubation Time on Total Protein (TP): Due to the denaturation of protein, the highest TP of the cystatin was recorded at an incubation time of 15 min for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). Further increases on the incubation time to 30, 45 and 60 min led to decreases in the TP of cystatin for all samples. Kathir et al. (2005) stated that the protein recovery depends on the incubation time and prolonged incubation can cause the protein to lose its native state. Khan and Bano (2008) reported that incubation of the sample after the alkaline treatment is necessary in order to get rid of the unwanted proteins present in the homogenate. In the present study, the TP of cystatin obtained after ammonium sulphate extraction showed a decrease of 73.8%, 83.9% and 81.7% for bovine lung, pancreas and mixture of lung and pancreas samples, respectively in comparison to TP obtained from crude extraction. The bovine lung sample recorded the highest TP.

The results obtained from the present study are comparable to those reported in the literature. Sadaf et al (2005) incubated the homogenate for 10 mins during the isolation of cystatin from goat kidney and reported a decrease of 82.5% in TP after ammonium sulphate extraction. NurhayatiNurhayati et al. (2013) studied the purification and characterization of cystatin (cathepsin inhibitor) from catfish and reported a decrease of 98.1% in TP after ammonium sulphate extraction from crude extract. Precipitation was performed at 10 mins of incubation. Sugo et al. (1981) reported a decrease of 99% in TP for cystatin extracted from horse plasma. Kirihara et al. (1995) reported a decrease of 99% in TP for cystatin extracted from bovine colostrum.

7.2.2.2. Effect of Incubation Time on Total Activity (TA): The highest TA was recorded at an incubation time of 30 min for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). Further increases in the incubation time led to

decreases in the TA. This is because the inadequate concentration of cystatin to counteract the increase in protease activity of papain and a total activity is always proportional to its concentration (Wilson et al., 2013). In the present study, the TA of cystatin obtained after ammonium sulphate extraction showed a decrease of 39.9%, 60.4% and 48.1% for bovine lung, pancreas and mixture of lung and pancreas samples, respectively on comparison to TA from crude extraction. The bovine lung sample recorded the highest TA due to high concentration of cystatin and high inhibitory activity of cysteine proteases in papain.

The results obtained from this study are comparable with those reported in the literature. Khan and Bano (2008) reported a decrease in TA of cystatin by 31.7% during the extraction of cystatin from goat lung compared to the TA of cystatin in the crude extract. Sumbul and Bano (2006) reported a decrease in TA of cystatin by 65.8% during the extraction of cystatin from goat brain sample compared to the TA obtained by crude extraction. Lee at al. (2008) reported a decrease of 64.1% in TA of cystatin extracted from pig plasma compared to the TA obtained from crude extract. Also, Kirikara et al. (1995) reported a decrease of 64.1% in TA of cystatin extracted from bovine colostrum compared to the TA obtained from crude extract.

7.2.2.3. Effect of Incubation Time on Specific Activity (SA): The highest SA was recorded at an incubation time of 30 min for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). Further increases on the incubation time led to decreases in the SA. In the present study, the SA of cystatin obtained after ammonium sulphate extraction showed an increase of 54.1% (from 0.111 to 0.172 Units/mg), 120% (from 0.007 to 0.016 Units/mg), and 230% (from 0.018 to 0.061 Units/mg) compared to SA from crude extraction for bovine lung, pancreas and mixture of lung and pancreas samples, respectively. The bovine lung sample recorded the highest SA due to high total protein content and total activity of cystatin. The extraction procedure enriched the cystatin fraction helping in high inhibition against target proteases (Koohmaraie and Kretchmar, 1990).

The results obtained from this study are comparable with those reported in the literature. Khan and Bano (2008) reported an increase in SA of cystatin of 4.3 % (from 0.206 to 0.215 Units/mg) during the extraction of cystatin from goat lung compared to the SA of cystatin in the crude extraction. Sumbul and Bano (2006) also reported an increase in SA of cystatin of 900% (from 0.015 to 0.15 Units/mg) during the extraction of cystatin from goat brain sample compared to SA obtained by crude extraction. Kirihara et al. (1995) reported a decrease of 4520% (from 25.8 to 1208 Units/mg) in SA of cystatin extracted from bovine colostrum.

7.2.2.4. Effect of Incubation Time on Yield (Y): The highest Y was recorded at an incubation time of 30 min for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). Further increases on the incubation time led to decreases in Y. In the present study, the Y of cystatin obtained after ammonium sulphate extraction were 60.0%, 39.5% and 51.8% for bovine lung, pancreas and mixture of lung and pancreas samples. The bovine lung sample showed the highest cystatin yield (Y) due to its high total activity and high specificity towards cysteine proteases.

These results obtained from the present study are comparable to those reported in the literature. Khan and Bano (2008) performed the extraction of cystatin (thiol proteinase inhibitor) from goat lung and reported a yield (Y) of 68% after extraction with ammonium sulphate using 30 min incubation time. Sumbul and Bano (2006) purified cystatins from goat brain and reported a yield (Y) of 31% after the extraction with ammonium sulphate at 15 min incubation. Baba et al. (2005) extracted cystatin from sheep plasma and reported a yield (Y) of 19.28 % after extraction with ammonium sulphate at 15 min incubation. Aghajanyan et al. (1988) reported a yield (Y) of 72.2% for cystatin extracted from bovine brain.

7.2.3. Effect of pH

The pH influences the solubility of the protein. The minimum solubility point of a protein is termed as isoelectric point. There is an optimum pH for individual proteins, at which the respective protein will be separated from other proteins (Bailey and Ollis,

1986). The solubility of protein depends on the salt concentration (Ladisch, 2001). Higher concentrations of salt provide a distinct electrostatic double-layer around the proteins, which makes the protein soluble. The solubility of protein is described by the following equation (Bailey and Ollis, 1986).

$$\ln (S) = K_S C_S \tag{7.1}$$

Where:

S = solubility of the protein (-)

K_S = salting out constant (m^3/kg-moles)

C_S = salt concentration (kg-moles/m^3).

In the present study, the effect of pH level (increasing pH from 6.5 to 8.0) on the TP (total protein), TA (total activity), specific activity (SA) and yield (Y) of cystatin at both saturations (25-45% and 45-65%) and various incubation times (15, 30, 45 and 60 min) from all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples) was variant. The change in pH changed the rate of enzyme catalysis. After incubation, the pH was adjusted to 7.5 in order to auto activate of the enzyme. Dickinson (2002) stated that the pH is important for yield and purity of cystatin. Shah and Bano (2010) stated that the stability of cystatin is influenced by the altering the net charge on the protein.

7.2.3.1. Effect of pH on Total Protein (TP): Increasing the pH from 6.5 to 7.5 increased the TP. Further increases in the pH to 8.0 decreased the TP of cystatin for all samples. The highest TP of the cystatin was recorded at the pH of 7.5 for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). The bovine lung sample recorded the highest TP due to its increased rate of catalysis caused by increased ionic strength. Improper pH levels leads to the loss of total protein content (Kopitar-Jerala, 2006).

The results obtained from the present study are comparable to those reported in the literature. Khan and Bano (2008) lowered the pH of the homogenate to 7.5 after incubation during their isolation of cystatin from goat lung and recovered the highest TP.

Priyadarshini and Bano (2009) used a pH of 7.5 and recovered the highest TP during the extraction of cystatin from goat pancreas. Baba et al. (2005) used a pH of 7.5 and recovered the highest TP of cystatin from sheep plasma. Berri et al. (1988) used a pH of 7.6 to recover the highest TP of cystatin from bovine skeletal muscle.

7.2.3.2. Effect of pH on Total Activity (TA): The highest TA was recorded at a pH of 7.5 for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). Increasing the pH from 6.5 to 7.5 increased the TA of cystatin, but further increases on the pH to 8.0 decreased the TA of cystatin for all samples. The bovine lung sample recorded the highest TA due to high concentration of cystatin and high inhibitory activity of cysteine proteases in papain (Kopitar-Jerala, 2006).

The results obtained from the present study are comparable to those reported in the literature. Sadaf et al (2005) lowered the pH of the homogenate to 7.5 after incubation during their isolation of cystatin from goat kidney and recovered the highest TA. Sumbul and Bano (2006) used a pH of 7.5 and recovered the highest TA during the extraction of cystatin from goat brain. Berri et al. (1988) used a pH of 7.6 and recovered the highest TA during the extraction of cystatin from bovine skeletal muscle. Synnes (1998) used a pH of 7.4 to recover the highest TA during the extraction of cystatin from Atlantic salmon.

7.2.3.3. Effect of pH on Specific Activity (SA): The highest SA was recorded at a pH of 7.5 for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). Increasing the pH from 6.5 to 7.5 increased the SA, but further increases on the pH to 8.0 decreased the SA of cystatin for all samples. The bovine lung sample recorded highest SA due to high concentration of cystatin enzyme activity.

The results obtained from the present study are comparable to those reported in the literature. Warwas and Sawicki (1988) isolated the cystatins from human placenta with a pH of 7.5 and produced a highest specific activity (SA) after fractionisation with ammonium sulphate. Shah et al (2012) used a pH of 7.5 for the chemical modification of goat liver cystatin and achieved maximal specific activity. Baba et al. (2005) used a pH

of 7.5 and recorded the highest SA of cystatin from sheep plasma. Peter et al. (1978) used a pH of 7.5 and recorded highest SA during extraction of cystatin from bovine nasal cartilage.

7.2.3.4. Effect of pH on Yield (Y): The highest Y was recorded at a pH of 7.5 for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). Increasing the pH from 6.5 to 7.5 increased the Y, but further increasing the pH to 8.0 decreased the Y of cystatin for all samples. The bovine lung sample recorded highest yield (Y) due to high activity of cystatin to inhibit the cysteine proteases in papain (Kopitar-Jerala, 2006).

The results obtained from the present study are comparable to those reported in the literature. Warwas and Sawicki (1988) isolated the cystatins from human placenta with a pH of 7.5 and produced a highest yield (Y) after fractionisation with ammonium sulphate. Also, Khan and Bano (2008) used a pH of 7.5 for the extraction of cystatin from goat lung and achieved highest yield. Kirihara et al. (1995) used a pH of 7.5 for the extraction of cystatin from bovine colostrum and produced the highest yield. Tsushima et al. (1995) used a pH of 8.0 and produced highest yield of cystatin from bovine hoof.

7.2.4. Effect of Ammonium Sulphate Saturation

In the present study, increasing the ammonium sulphate saturation from 25-45% to 45-65% produced variable results in the TP (total protein), TA (total activity), specific activity (SA) and yield (Y) of cystatin from all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples) at all incubation times (15, 30, 45 and 60 min) and pH levels (6.5, 7.0, 7.5 and 8.0). Changing the salt concentration changed the protein solubility leading to better cystatin yield. The increase in salt concentration (decreasing the protein solubility) is referred as salting-out, which creates protein-protein interactions that result in higher cystatin concentration (Bailey and Ollis, 1986).

7.2.4.1. Effect of Ammonium Sulphate Saturation on Total Protein (TP): The highest TP of the cystatin was recorded with the 45-65% ammonium sulphate saturation for all

the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). Ammonium sulphate is an anti-chaotropic salt as it exposes the hydrophobic residues on the surface of protein layer to the salt. The salt sheds the highly structured water layer around the protein leading to the precipitate formation. The bovine lung sample recorded the highest TP because it had an effective hydrophobic reaction with the ammonium sulpahte (Gosh, 2006).

The results obtained from the present study are comparable to those reported in the literature. Rawdkuen et al (2005) studied the characteristics of cystatin from chicken plasma and reported a total protein recovery with 40% ammonium sulphate extraction. Nurhayati et al (2013) performed the purification of cathepsin inhibitor from catfish with 30-80% saturation and recovered highest total protein content. Aghajanyan et al. (1988) used a saturation of 40-70% for the isolation of cystatin from bovine brain.

7.2.4.2. Effect of Ammonium Sulphate Saturation on Total Activity (TA): The highest TA of the cystatin was recorded with 45-65% ammonium sulphate saturation for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). The bovine lung sample recorded the highest TA. This is because of the electrostatic charge provided around the protein by the salt concentration with increased protein-protein interaction leading to increased activity (Gosh, 2006).

The results obtained from the present study are comparable to those reported in the literature. Sadaf et al (2005) isolated cystatin from goat kidneys using an ammonium sulphate saturation of 50-80% and achieved a high total activity (TA) of cystatin. Priyadarshini and Bano (2009) extracted cystatins from goat pancreas with 20-80% ammonium sulphate saturation and obtained high total activity (TA). Baba et al. (2005) used an ammonium sulphate saturation of 40-60% for the extraction of cystatin from sheep plasma. Nuhayati et al. (2013) used an ammonium sulphate saturation of 30-80% for the extraction of cystatin from catfish.

7.2.4.3. Effect of Ammonium Sulphate Saturation on Specific Activity (SA): The highest SA of the cystatin was recorded with 45-65% the ammonium sulphate saturation for all the three samples (bovine lung, pancreas and mixture of lung and pancreas

samples). The bovine lung sample recorded the highest SA. This was because of increased cystatin concentration by the high protein-protein interaction (Bailey and Ollis, 1986).

The results obtained from the present study are comparable to those reported in the literature. Khan and Bano (2008) used 40-60% saturation for isolating cystatins from goat lung and reported high specific activity. Sadaf et al (2005) isolated cystatins from goat kidneys, used an ammonium sulphate saturation of 50-80% and reported high specific activity of cystatin. Aghajanyan et al. (1988) used a saturation of 40-70% for achieving the highest SA of cystatin from bovine brain. Rawdkuen et al. (2005) used an ammonium sulphate saturation of 40% for the extraction of cystatin form chicken plasma.

7.2.4.4. Effect of Ammonium Sulphate Saturation on Yield (Y): The highest Y of the cystatin was recorded with 45-65% the ammonium sulphate saturation for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). The bovine lung sample recorded highest Y due to increased inhibitory activity of cystatin against the cysteine proteases in papain (Kopitar-Jerala, 2006).

The results obtained from the present study are comparable to those reported in the literature. Rawdkuen et al (2005) studied the characteristics of cysteine proteinase inhibitor from chicken plasma. Both PEG fractionisation and ammonium sulphate fractionation was performed to determine the method with high yield. The ammonium sulphate precipitation performed at 40% saturation fetched higher cystatin yield than the PEG method. Nuhayati et al (2013) performed the purification of cathepsin inhibitor from catfish with 30-80% saturation and reported high cystatin yield. Baba et al. (2005) used an ammonium sulphate saturation of 40-60% for the extraction of cystatin from sheep plasma and recovered the highest yield. Aghajanyan et al. (1988) used a saturation of 40-70% for the extraction of cystatin from bovine brain and reported the highest yield.

7.2.5. Optimum conditions for Cystatin by Ammonium Sulphate Extraction

The optimum controls for the extraction of cystatin from all the three samples (bovine lung sample, pancreas sample, lung and mixture of pancreas sample) are shown in Table 7.1. The recommended parameter values for all the three samples (bovine lung sample, pancreas sample, lung and mixture of pancreas sample) are an incubation time 30 min, a pH of 7.5 and an ammonium sulphate saturation of 45-65%.

7.3. Effect of pH and Temperature on the Maximal inhibitory activity

The effects of pH (3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5 and 11.5) and temperature (30, 40, 50, 60, 70, 80, and 90 ºC) on the maximal inhibitory activity of cystatin obtained from all the three samples (bovine lung, pancreas, mixture of lung and pancreas samples) were studied. The caseinolytic method was used for the evaluation of cystatin inhibitory activity for all the three samples (bovine lung, pancreas and mixture of lung and pancreas samples). Increasing the pH level from 3.0 to 7.5 resulted increases in the inhibitory activity of cystatin and further increase in pH led to decrease of inhibitory activity.

Increasing temperature from 30 to 50 ºC resulted in maximal inhibitory activity, whereas further increase led to decrease in activity. Very low and extremely higher pH and temperature levels show lower inhibitory activity of cystatin. The optimal pH and temperature for maximal inhibitory activity were 7.5 and 50 ºC for all samples (bovine lung sample, pancreas sample, lung and mixture of pancreas sample).

Caesin is more resistant to the hydrolytic action of alkali than the other proteins (Folin and Ciocalteu, 1927). Caseinolytic activity was achieved by the ability of an enzyme to liberate titrable carboxyl groups on their reaction with casein. Casein acts as substrate in the reaction.

The rate of digestion of casein by the enzyme determines the enzyme activity. The rate of digestion of casein was affected by pH and temperature and hence optimization of these factors leads to an efficient inhibitor activity.

Table 7.1. Optimal conditions for extraction of cystatin from bovine lung sample, bovine pancreas sample and mixture of bovine lung and pancreas sample.

Parameters	Optimum Controlled		
	Incubation time (mins)	pH	Ammonium sulphate saturation
TP (mg)	15	7.5	45-65%
TA (Units)	30	7.5	45-65%
SA (Units/mg)	30	7.5	45-65%
Y (%)	30	7.5	45-65%

TP : Total protein content
TA : Total inhibitory activity
SA : Specific activity
Y : Yield

Chow and Peticolas (1948) stated that the rate of digestion of casein is proportional to the enzyme activity. Several researchers reported similar results (Rawdkuen et al, 2005; Nuhayati et al., 2013; Aghajanyan et al., 1988; Sadaf et al., 2005; Priyadarshini and Bano, 2009; Khan and Bano, 2008).

Rawdkuen et al (2005) fractionated cystatins from chicken plasma and optimized the pH and temperature for maximal inhibitory activity. BANA (N_{α}-Benzoyl-DL-arginine-2-naphthylamide hydrochloride) was used as substrate to determine the inhibitory activity. The optimal pH and temperature for maximal inhibitory activity were 8.0 and 90 ºC, respectively.

Nuhayati et al (2013) optimized the pH and temperature for maximal inhibitory activity during the isolation of cystatin from catfish. The assay was performed using hemoglobin as substrate to determine the inhibitory activity. The optimal pH and temperature were found to be 8.0 and 40 ºC, respectively.

Aghajanyan et al. (1988) isolated cystatins from bovine brain and optimized the temperature for maximal inhibitory activity. P-Pxy-Hb was used as substrate for determining the inhibitory activity. The optimal temperature was 80 ºC.

Priyadarshini and Bano (2009) used casein as substrate for determining inhibitor activity during the extraction of cystatin from goat pancreas. The optimal pH and temperature for maximal inhibitor activity were found to be 7.5 and 60 ºC, respectively.

Khan and Bano (2008) used casein for determining inhibitory activity of cystatin during the extraction of cystatin from goat lung. The optimal pH for maximal inhibitor activity was found to be 7.0 - 8.0.

7.2.6 Comparative analysis and recommendation

Table.7.1. shows a comparative analysis of TP, TA, SA and Y of cystatin obtained from all the three samples (bovine lung sample, pancreas sample, lung and mixture of pancreas sample). The result showed the presence of cystatins in both bovine lung and bovine pancreas sample. The lung sample has more total activity and specificity to inhibit

Table 7.2. Comparing analysis of bovine lung sample, bovine pancreas sample and mixture of bovine lung and pancreas sample at optimum condition after ammonium sulphate fractionation (n=3).

Sample Type	Total Weight (g)	TP		TA		SA	
		(mg/g)	Total (mg)	(units/g)	Total(units)	(units/g)	Total (units)
Lung	655	13.94	9130.7	2.5	1637.5	0.0009	0.56
Pancreas	350	11.07	3874.5	0.1	35.0	0.0001	0.03
Mixture of bovine lung and pancreas	1005	12.63	12693.1	0.7	703.5	0.0003	0.30

Replicates =3
TP : Total protein content
TA : Total inhibitory activity
SA : Specific activity
Y : Yield

cysteine proteases than the bovine pancreas and mixture of lung and pancreas samples. Hence it will be more efficient to separate the lung from the bovine pancreas during slaughterhouse processing to recover better cystatin yield.

CHAPTER 8. RECOMMENDATIONS

1. The characterization of cystatin like groups and the kinetics should be investigated.

2. Purification techniques for cystatins should be explored and compared.

3. The stability of cystatins at different pH's and temperatures in the assay should be evaluated.

4. The effects of various metal ions and oxidizing agents on cystatin inhibitory activity should be examined.

5. During the slaughtering process, it will be more efficient to separate the lung from the bovine pancreas in order to recover better cystatin yield.

6. The results from this study should be scaled up as it plays a major role in waste management and production of valuable product.

CHAPTER 9. CONCLUSIONS

The study involved the isolation of cystatins from three samples (included bovine lung, pancreas and mixture of lung and pancreas samples). The effects of incubation time (15, 30, 45 and 60 min), pH (6.5, 7.0, 7.5 and 8.0) and ammonium sulphate saturation (25-45% and 45-65%) on TP, TA, SA and Y were studied. The following conclusions can be made.

1. The incubation time had significant effect on TP, TA, SA and Y at the 0.001 level.
   (a) A 30 min incubation period provided the highest yield for all the three samples.
   (b) The highest TP was recovered at an incubation time of 15 min. Further increase in incubation time above 30 mins decreased the TP as a result of unwanted protein denaturation.
   (c) The TA, SA and Y highest values were obtained at the optimal incubation time of 30 mins. Further increase in incubation time above 30 min decreased their values.
   (d) Among the three samples, bovine lung sample provided the highest TP, TA, SA and Y at the optimal incubation time (30 mins).

2. The pH had significant effect on TP, TA, SA and Y at 0.001 level.
   (a) Increase in pH from 6.0 to 7.5 increased the TP, TA, SA and Y. Further increase in pH to 8.0 decreased their values for all the three samples.
   (b) The optimal pH range (7.5) helps in the activation of cystatin and production of high cystatin yield.
   (c) Among the three samples, bovine lung sample recorded the highest TP, TA, SA and Y of cystatin at the optimal pH of 7.5.

3. The ammonium sulphate saturation had significant effect on TP, TA, SA and Y at 0.001 level.
   (a) The increase in ammonium sulphate saturation from 25-45% to 45-65% increased the TP, TA, SA and Y.

(b) The yield recovered at 25-45% ammonium sulphate saturation with optimal conditions of incubation time (30 mins) and pH (7.5) were 52.12, 36.93, and 45.61% for bovine lung, pancreas and mixture of lung and pancreas samples respectively.

(c) The yield recovered at 45-65% ammonium sulphate saturation with optimal conditions of incubation time (30 mins) and pH (7.5) were 60.07, 39.57 and 51.89% for bovine lung, pancreas and mixture of lung and pancreas samples respectively.

(d) 45-65% ammonium sulphate saturation produced better yield than 25-45% at all incubation times (15, 30, 45 and 60 min) and pH levels (6.5, 7.0, 7.5 and 8.0) for all the three samples.

(e) The bovine lung sample provided the highest TP, TA, SA and Y at 45-65% ammonium sulphate saturation.

4. The two way, three way and four way interaction between the incubation time, pH and saturation % were significant for all three sample at 0.001 level.

5. The optimal conditions required for extraction of cystatins from bovine lung sample, pancreas and mixture of bovine lung and pancreas samples were similar.

6. The bovine lung sample produced the highest cystatin yield (60.07%), compared to the bovine pancreas (39.57%) and mixture of lung and pancreas (51.89%) samples.

7. The optimum pH and temperature in the inhibitory assay for maximal inhibitory activity were 7.5 and 50 ºC for all three samples.

8. The bovine lung sample exhibited high inhibitory activity of 55.1% at the optimized conditions.

9. The bovine lung sample produced the highest values in TP, TA, SA, Y.

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APPENDIX A

CRUDE EXTRACTION OF CYSTATIN FROM BOVINE LUNG, PANCREAS AND MIXTURE OF LUNG AND PANCREAS SAMPLES

+	: After homogenization
++	: After adjusting the pH to 11
Sample size	= 200g
Replicates	= 3
TP	: Total protein content
TA	: Total inhibitory activity
SA	: Specific activity
Y	: Yield

Table A1: Crude extraction of cystatin from lung, pancreas and mixture of lung and pancreas

Bovine sample	Step	Volume (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
Lung	Crude[+]	400	10651	842.3	0.087	100.00
		400	10656	844.5	0.087	100.00
		400	10658	841.9	0.086	100.00
	Average	400±0.00	10655±0.63	842.9±0.39	0.087±0.008	100.00
	Alkaline treatment (pH 11)[++]	380	7563	760.9	0.101	90.30
		395	7566	762.1	0.101	90.40
		380	7578	758.5	0.100	90.00
	Average	385±0.98	7569±0.93	760.9±0.45	0.100±0.008	90.3±0.15
Pancreas	Crude [+]	350	13783	74.1	0.005	100.00
		365	13787	76.8	0.006	100.00
		365	13788	75	0.005	100.00
	Average	400±0.98	10655±0.54	75.3±0.39	0.005±0.008	100.00±0.0
	Alkaline treatment (pH 11) [++]	350	10241	60.3	0.006	80.07
		365	10248	60.1	0.006	79.81
		365	10237	60.1	0.006	79.81
	Average	360±0.98	10242±0.78	60.3±0.36	0.006±0.003	79.90±0.42

Table A1: Continued.

Bovine Sample	Step	Volume (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
Lung and pancreas mixture	Crude[+]	400	20355	296.6	0.015	100.00
		400	20355	297.3	0.015	100.00
		400	20358	299.8	0.015	100.00
	Average	400±0.00	10655±0.43	297.9±0.43	0.015±0.00	100.00±0.00
	Alkaline treatment (pH 11)[++]	370	13833	247.3	0.018	83.00
		375	13835	248.1	0.018	83.30
		365	13837	244.4	0.018	82.00
	Average	370±0.74	13835±0.47	246.6±0.46	0.018±0.00	82.79±0.28

APPENDIX B

EXTRACTION OF CYSTATIN FROM BOVINE LUNG SAMPLE WITH AMMONIUM SULPHATE

TA = Total inhibitory activity (Units)

SA = specific activity (Units/mg)

TP = Total protein (mg)

Y = Yield (%)

Replicates = 3

Table B1: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from bovine lung at an incubation time of 15 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
15	6.5	25-45%	212.0	2250	200.0	0.089	23.7
			210.0	2252	205.0	0.091	24.3
			209.5	2251	204.0	0.091	24.2
		Average	210.5±0.38	2251.0±0.33	203.0±0.54	0.090±0.011	24.08±0.19
		45-65%	210.0	2555	250.0	0.098	29.7
			210.0	2558	254.0	0.099	30.2
			210.0	2558	255.0	0.100	30.3
		Average	210.0±0.00	2557.0±0.43	253.0±0.56	0.099±0.010	30.01±0.19
	7.0	25-45%	210.0	2675	262.0	0.098	31.1
			210.0	2677	265.0	0.099	31.5
			210.0	2680	268.6	0.100	31.9
		Average	210.0±0.00	2677.3±0.52	265.2±0.60	0.099±0.011	31.43±0.21
		45-65%	208.5	2960	386.0	0.130	45.8
			208.5	2968	387.2	0.130	45.9
			207.0	2964	387.5	0.131	46.0
		Average	208.0±0.31	2964.0±0.66	386.9±0.29	0.131±0.004	45.79±0.10

Table B1: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
15	7.5	25-45%	210.0	2755	370.5	0.134	44.0
			210.0	2758	370.0	0.134	43.9
			211.5	2757	371.6	0.135	44.1
		Average	210.5±0.31	2756.7±0.41	370.7±0.30	0.134±0.006	43.97±0.10
		45-65%	212	3030	450	0.149	53.4
			210	3040	452	0.149	53.7
			209.5±0.21	3039	448.3	0.148	53.2
		Average	210.5	3036.3±5.90	450.1±0.17	0.148±0.0003	53.39±0.010
	8.0	25-45%	208.0	1886	155.5	0.082	18.5
			210.0	1888	156.0	0.083	18.5
			213.5	1897	157.4	0.083	18.7
		Average	210.5±0.55	1890.3±0.85	156.3±0.33	0.083±0.005	18.54±0.11
		45-65%	207.5	2248.0	200.0	0.089	23.7
			210.0	2248.8	205.0	0.091	24.3
			214.0	2252.3	201.9	0.090	24.0
		Average	210.5±0.60	2249.7±1.85	202.3±0.55	0.090±0.0006	24.00±0.05

Table B2: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from bovine lung at an incubation time of 30 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
30	6.5	25-45%	212.5	2065	270	0.131	32.1
			212.0	2070	275.5	0.133	32.7
			211.5	2074.1	283.7	0.137	33.7
		Average	212.0±0.23	2069.7±0.71	276.4±0.87	0.134±0.018	32.79±0.30
		45-65%	212.0	2160	292.5	0.135	34.7
			210.0	2168	296	0.137	35.1
			209.5	2167	297.7	0.137	35.3
		Average	210.5±0.38	2165.0±0.69	295.4±0.54	0.140±0.01	35.04±0.19
	7.0	25-45%	210.0	2160	311	0.144	36.9
			212.0	2168	315	0.145	37.4
			208.0	2271.1	309.4	0.136	36.7
		Average	210.0±0.31	2199.7±7.33	311.8±1.76	0.142±0.0005	36.99±1.90
		45-65%	210.0	2560	425.5	0.166	50.5
			210.0	2562	426.2	0.166	50.6
			210.0	2562	422.7	0.165	50.2
		Average	210.0±0.00	2561.3±0.35	424.8±0.45	0.166±0.009	50.39±0.16

Table B2: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
30	7.5	25-45%	210.5	2548	439.8	0.173	52.2
			212.0	2550	440.5	0.173	52.3
			213.5	2549.6	437.9	0.172	52.0
		Average	212.0±0.40	2549.2±0.34	439.4±0.38	0.163±0.008	52.12±0.13
		45-65%	210.0	2785	508.0	0.182	60.3
			210.0	2789	505.5	0.181	60.0
			211.5	2792	503.9	0.181	59.8
		Average	210.5±0.31	2788.7±0.57	505.8±0.47	0.172±0.010	60.07±0.17
	8.0	25-45%	210.0	1710	188.2	0.118	22.3
			210.0	1719	190.0	0.118	22.6
			211.5	1716	187.9	0.117	22.3
		Average	210.5±0.31	1715.1±0.73	188.7±0.56	0.118±0.010	22.38±0.06
		45-65%	212.0	1925	235	0.1221	27.9
			207.0	1932	236	0.1222	28
			212.5	1933	240	0.1242	28.5
		Average	210.0±0.58	1930.0±0.69	237.0±0.54	0.130±0.011	28.11±0.19

Table B3: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from bovine lung at an incubation time of 45 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
45	6.5	25-45%	210.5	1786	235.0	0.121	27.8
			210.0	1788	236.0	0.121	28.0
			209.5	1796	246.9	0.127	29.3
		Average	210.0±0.23	1790.0±0.76	239.3±0.85	0.122±0.019	23.35±0.30
		45-65%	210.5	1989.2	252.5	0.133	30.0
			210	1988	250.8	0.131	29.8
			209.5	1982.9	251.5	0.133	29.9
		Average	210.9±0.23	1986.7±0.60	251.6±0.30	0.133±0.006	29.84±0.11
	7.0	25-45%	212.0	1965	270.5	0.137	32.1
			207.0	1962	276.0	0.140	32.8
			212.5	1953	276.4	0.141	32.8
		Average	210.0±0.58	1960.0±2.50	274.3±3.13	0.136±0.0015	30.54±0.35
		45-65%	212.0	2095.2	293.5	0.150	34.8
			210.0	2090.5	294.0	0.151	34.9
			209.5	2104.4	286.7	0.156	34
		Average	210.5±0.38	2096.7±0.38	291.4±0.50	0.154±0.0003	34.57±0.05

Table B3: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
45	7.5	25-45%	210.0	205	293.5	0.143	34.8
			210.0	2058	294.0	0.143	34.9
			211.5	2057	343.7	0.157	40.8
		Average	210.5±0.31	2055.3±0.67	310.4±1.79	0.151±0.039	36.82±0.62
		45-65%	210.5	2050	329.5	0.151	39.1
			212.0	2058	332.0	0.151	39.4
			213.5	2530	325.5	0.159	38.6
		Average	212.0±0.40	2213.0±0.52	329.0±0.60	0.156±0.046	39.03±0.21
	8.0	25-45%	212.0	1609	178.5	0.107	21.2
			207.0	1605	179.8	0.110	21.3
			212.5	1614	181.1	0.107	21.5
		Average	210.0±0.58	1609.7±0.80	179.8±0.49	0.108±0.0012	21.33±0.35
		45-65%	210.0	1655	205.6	0.124	24.4
			212.0	1655	208.9	0.126	24.8
			208.0	1652	206.8	0.125	24.6
		Average	210.0±0.31	1654.1±3.68	207.1±6.40	0.125±0.0035	23.56±0.68

Table B4: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from bovine lung at an incubation time of 60 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
60	6.5	25-45%	212.5	1559	155.2	0.100	18.4
			212.0	1558	154.8	0.099	18.4
			211.5	1557	152.6	0.098	18.1
		Average	212.0±0.23	1558.3±0.80	154.2±1.15	0.099±0.0009	18.29±0.07
		45-65%	212.0	1656	185.3	0.116	22.0
			210.0	1652	186.2	0.117	22.1
			209.5	1646	182.3	0.120	21.6
		Average	210.5±0.38	1651.6±2.33	184.6±0.72	0.119±0.0006	21.90±0.05
	7.0	25-45%	210.0	1658	202.2	0.122	24
			212.0	1656	205.3	0.124	24.4
			208.0	1653	201.2	0.122	23.9
		Average	210.0±0.31	1655.7±2.03	202.9±0.46	0.123±0.0003	24.07±0.08
		45-65%	210.0	1820	242.2	0.143	28.8
			210.0	1816	245.3	0.145	29.1
			210.0	1822	257.7	0.141	30.6
		Average	210.0±0.00	1819.7±0.54	248.4±0.77	0.144±0.0003	29.46±0.07

Table B4: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
60	7.5	25-45%	210.0	1715	267.0	0.156	31.7
			210.0	1719	271.0	0.158	32.2
			211.5	1716.4	269.0	0.157	31.9
		Average	210.5±0.31	1717.2±1.14	269.0±0.64	0.157±0.0015	31.91±0.14
		45-65%	212	1986	295.3	0.149	35.1
			210	1985	297.1	0.150	35.3
			210.5	1988	292.6	0.147	34.7
		Average	209.5±0.21	1986.9±0.44	295.0±0.50	0.148±0.012	34.99±0.17
	8.0	25-45%	208.0	1419	144.5	0.102	17.2
			210.0	1418	145.5	0.103	17.3
			213.5	1418	142.0	0.100	16.9
		Average	210.5±0.55	1418.4±1.13	144.0±0.15	0.102±0.003	16.08±0.02
		45-65%	210.0	1458	163.5	0.112	19.4
			212.0	1457	166.1	0.114	19.7
			208.0	1459	162.4	0.111	19.3
		Average	210.0±0.31	1458.8±0.33	164.0±0.45	0.112±0.012	17.22±0.16

APPENDIX C

EXTRACTION OF CYSTATIN FROM BOVINE PANCREAS SAMPLE WITH AMMONIUM SULPHATE

TA = Total inhibitory activity (Units)

SA = specific activity (Units/mg)

TP = Total protein (mg)

Y = Yield (%)

Replicates = 3

Table C1: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from bovine pancreas at an incubation time of 15 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
15	6.5	25-45%	175.0	1856	14.6	0.008	19.7
			178.0	1858	14.9	0.008	18.7
			173.5	1853	13.7	0.007	17.6
		Average	175.5±0.38	1855.9±0.45	14.4±0.05	0.007±0.0000	19.12±0.06
		45-65%	176.0	1958	17.5	0.009	23.2
			173.5	1956	16.9	0.009	22.4
			175.5	1954	17.2	0.009	22.8
		Average	175.0±0.35	1956.0±0.47	17.2±0.18	0.009±0.004	22.84±0.08
	7.0	25-45%	178.5	1953.9	18.5	0.009	23.2
			178	1952.6	19.9	0.009	25.0
			179	1953.4	20.8	0.011	24.9
		Average	178±0.23	1953.3±0.26	18.4±0.48	0.010±0.011	24.47±0.17
		45-65%	175.0	2099	23.9	0.011	31.7
			175.0	2100	22.4	0.011	29.7
			175.0	2100	22.1	0.011	29.3
		Average	175.0±0.00	2100.3±0.22	22.8±0.32	0.011±0.007	30.31±0.38

Table C1: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
15	7.5	25-45%	175.0	2199	26.5	0.012	35.2
			175.0	220	27.6	0.013	36.7
			175.0	219	26.0	0.012	34.5
		Average	175.0±0.00	2199.3±0.33	26.7±0.30	0.011±0.006	35.45±0.35
		45-65%	175.0	2576	29.5	0.011	39.2
			176.0	2572	28.8	0.011	38.2
			175.5	2573	28.4	0.011	37.7
		Average	175.5±0.23	2573.7±0.48	28.9±0.24	0.012±0.005	38.37±0.29
	8.0	25-45%	175.0	1629	8.3	0.006	11.0
			178.0	1630	8.6	0.006	11.4
			173.5	1627.1	7.1	0.006	9.43
		Average	175.5±0.15	1628.7±0.40	8.0±0.29	0.006±0.000	10.67±0.34
		45-65%	175.0	1675	8.3	0.005	11
			178.0	1675	8.6	0.005	11.4
			173.5	1669	7.7	0.005	10.2
		Average	175.5±0.11	1673.3±0.64	8.2±0.22	0.008±0.005	10.90±0.26

Table C2: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from bovine pancreas at an incubation time of 30 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
30	6.5	25-45%	176.0	1744	16.8	0.010	22.3
			173.5	1745	17.3	0.010	23.0
			175.5	1739	15.1	0.009	20.1
		Average	175.0±0.35	1743.2±0.62	16.4±0.35	0.009±0.008	21.73±0.41
		45-65%	175.0	1759	19.9	0.011	26.4
			178.0	1759	20.5	0.012	27.2
			173.5	1757	18.4	0.010	24.4
		Average	175.5±0.15	1758.7±0.34	19.6±0.34	0.012±0.008	25.57±0.40
	7.0	25-45%	175.0	1889	20.9	0.011	27.8
			176.0	1888	21.2	0.011	28.2
			175.5	1885	18.5	0.010	24.6
		Average	175.5±0.23	1887.8±0.45	20.2±0.55	0.011±0.009	26.73±0.47
		45-65%	176.0	1988	24.8	0.012	32.9
			173.5	1988	26.6	0.013	35.3
			175.5	1982	21.2	0.011	28.2
		Average	175.0±0.35	1986.3±0.59	24.2±0.55	0.013±0.012	32.01±0.64

Table C2: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
30	7.5	25-45%	175.0	2055	28.4	0.014	37.7
			178.0	2056	26.8	0.013	35.6
			173.5	2051	28.2	0.014	37.5
		Average	175.5±0.23	2054.1±0.52	27.8±0.31	0.014±0.07	36.93±0.36
		45-65%	176.0	2215	28.4	0.018	37.7
			173.5	2216	26.8	0.013	35.6
			175.5	2213	34.2	0.015	45.4
		Average	175.0±0.35	2214.7±0.40	29.8±0.65	0.016±0.01	39.57±0.76
	8.0	25-45%	175.0	1531	9.2	0.006	12.2
			178.0	1530	10.3	0.007	13.7
			173.5	1526	9.6	0.006	12.7
		Average	175.5±0.15	1529.1±0.54	9.7±0.19	0.010±0.006	12.91±0.29
		45-65%	175.0	1636	13.8	0.008	18.3
			175.0	1638	11.1	0.007	14.7
			175.0	1629	14.7	0.009	19.5
		Average	175.0±0.00	1634.4±0.71	13.2±0.45	0.008±0.011	17.49±0.53

Table C3: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from bovine pancreas at an incubation time of 45 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
45	6.5	25-45%	175.0	1454	13.8	0.009	18.3
			178.0	1452	14.1	0.010	18.7
			173.5	1453	12.9	0.009	17.1
		Average	175.5±0.38	1453.0±0.33	13.60±0.26	0.008±0.007	18.06±0.30
		45-65%	176.0	1616	15.42	0.009	20.4
			173.5	1615	16.20	0.010	21.5
			175.5	1616	13.77	0.008	18.2
		Average	175.0±0.35	1615.7±2.60	15.13±0.37	0.010±0.009	20.09±0.43
	7.0	25-45%	178.5	1624	17.8	0.011	23.6
			178	1624	18.5	0.010	24.6
			179	1618	16.59	0.010	22.0
		Average	178±0.23	1622.0±0.62	17.63±0.32	0.010±0.008	23.41±0.38
		45-65%	175.0	1650	21.8	0.013	29.0
			175.0	1652	22.1	0.013	29.3
			175.0	1647	19.4	0.012	25.8
		Average	175.0±0.00	1649.7±0.52	21.10±0.40	0.012±0.001	28.02±0.47

Table C3: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
45	7.5	25-45%	176.0	1692	20.2	0.012	26.8
			173.5	1694	21.8	0.013	29
			175.5	1689	20.91	0.012	27.8
		Average	175.0±0.35	1691.7±0.52	20.97±0.29	0.012±0.007	27.84±0.34
		45-65%	175.0	1886	25.8	0.014	34.3
			178.0	1886	25.1	0.013	33.3
			173.5	1885	21.49	0.011	28.5
		Average	175.5±0.15	1885.7±0.24	24.13±0.50	0.013±0.012	32.04±0.58
	8.0	25-45%	175.0	1374	8.50	0.006	11.3
			175.0	1376	7.20	0.005	9.56
			175.0	1374	8.39	0.006	11.1
		Average	175.0±0.00	1374.7±0.35	8.03±0.28	0.007±0.08	10.67±0.33
		45-65%	175.0	1489	10.5	0.009	13.9
			175.0	1490	11.4	0.008	15.1
			175.0	1485.9	10.53	0.010	14
		Average	175.0±0.00	1488.3±0.48	10.81±0.28	0.009±0.006	13.62±0.27

Table C4: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from bovine pancreas at an incubation time of 60 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
60	6.5	25-45%	176.0	1333	6.8	0.005	9.03
			173.5	1329	6.5	0.005	8.63
			175.5	1328	6.26	0.005	8.31
		Average	175.0±0.35	1330.3±2.60	6.52±0.42	0.005±0.003	8.66±0.12
		45-65%	175.0	1421	8.8	0.006	11.7
			178.0	1423	7.6	0.005	10.1
			173.5	1416	8.77	0.006	11.6
		Average	175.5±0.15	1420.0±0.40	8.39±0.42	0.007±0.003	11.15±0.45
	7.0	25-45%	175.0	1505	14.2	0.009	18.9
			176.0	1503	13.8	0.009	18.3
			175.5	1506	11.9	0.008	15.8
		Average	175.5±0.23	1504.7±0.33	13.30±0.17	0.009±0.003	17.66±0.24
		45-65%	176.0	1526	15.8	0.004	21
			173.5	1528	14.9	0.004	19.8
			175.5	1522	14.9	0.004	19.8
		Average	175.0±0.35	1525.3±0.36	15.20±0.17	0.004±0.000	20.18±0.50

Table C4: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
60	7.5	25-45%	175.0	1557	15.55	0.010	20.7
			178.0	1558	16.40	0.010	21.8
			173.5	1556	15.24	0.010	20.2
		Average	175.5±0.23	1557.3±0.26	15.73±0.23	0.010±0.000	20.88±0.24
		45-65%	176.0	1627	17.9	0.011	23.8
			173.5	1628	18.4	0.011	24.4
			175.5	1625	16.5	0.01	21.9
		Average	175.0±0.35	1626.7±0.49	17.60±0.50	0.011±0.003	23.37±0.38
	8.0	25-45%	175.0	1255	6.80	0.005	9.03
			178.0	1256	6.50	0.005	8.63
			173.5	1252	5.72	0.005	7.60
		Average	175.5±0.15	1254.3±0.48	6.34±0.24	0.005±0.007	7.42±0.09
		45-65%	175.0	1294	8.10	0.006	10.8
			175.0	1295	8.60	0.007	11.4
			175.0	1287	7.96	0.006	10.6
		Average	175.0±0.00	1292.0±0.40	8.22±0.120	0.006±0.000	10.91±0.13

APPENDIX D

EXTRACTION OF CYSTATIN FROM MIXTURE OF BOVINE LUNG AND PANCREAS WITH AMMONIUM SULPHATE

TA = Total inhibitory activity (Units)

SA = specific activity (Units/mg)

TP = Total protein (mg)

Y = Yield (%)

Replicates = 3

Table D1: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from mixture of lung and pancreas at an incubation time 15 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
15	6.5	25-45%	212.0	1960	65.4	0.033	22
			210.0	1958	63.7	0.033	21.4
			209.5	1953	63.9	0.033	21.4
		Average	210.5±0.38	1957.3±0.63	64.33±0.32	0.033±0.007	21.59±0.19
		45-65%	210.0	2282	78.4	0.034	26.3
			210.5	2284	79.3	0.035	26.6
			217.0	2276	78.8	0.035	26.5
		Average	212.5±0.65	2280.9±0.64	78.87±0.22	0.035±0.005	26.47±0.13
	7.0	25-45%	209.0	2315	95.8	0.041	32.2
			209.5	2316	95.8	0.041	32.2
			213.0	2314	94.6	0.041	31.8
		Average	210.5±0.49	2315.0±0.33	95.40±0.27	0.041±0.006	32.02±0.16
		45-65%	209.0	2462	116.5	0.047	39.1
			213.0	2460	117.8	0.048	39.5
			214.0	2461	113.7	0.046	38.2
		Average	212.3±0.54	2461.2±0.34	116.00±0.48	0.048±0.001	38.61±0.28

Table D1: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
15	7.5	25-45%	210.0	2650.1	118.8	0.045	39.9
			210.5	2649.8	117.9	0.044	39.6
			215.5	2648.6	118.8	0.045	39.9
		Average	212.0±0.58	2649.5±0.29	118.50±0.24	0.045±0.005	39.77±0.14
		45-65%	212.0	2782	128.4	0.046	43.1
			212.0	2783	126.7	0.046	42.5
			213.5	2782	127.7	0.046	42.9
		Average	212.5±0.31	2782.8±0.26	127.60±0.30	0.046±0.006	42.83±0.18
	8.0	25-45%	210.0	1782	46.8	0.026	15.7
			211.5	1785	46.2	0.026	15.5
			210.0	1782	45.9	0.026	15.4
		Average	210.5±0.58	1783.7±3.49	46.30±0.16	0.026±0.0000	15.52±0.04
		45-65%	211	1995	63.2	0.032	21.2
			213.5	1995	62.5	0.031	21.0
			213.0	1994	62.7	0.031	21.0
		Average	212.5±0.38	1995.2±0.19	62.80±0.20	0.032±0.004	21.10±0.12

Table D2: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from mixture of lung and pancreas at an incubation time 30 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
30	6.5	25-45%	212.0	1832	82.8	0.045	27.79
			212.0	1831	81.6	0.044	27.39
			212.0	1833.3	83.1	0.045	27.89
		Average	212.0±0.0	1832.1±1.72	82.55±0.45	0.044±0.000	27.71±0.13
		45-65%	212.0	1895	89.5	0.045	31.0
			212.0	1895	89.9	0.045	30.2
			213.5	1892	88.8	0.045	29.8
		Average	212.5±0.318	1894.0±43	89.41±0.24	0.045±0.005	31.01±0.13
	7.0	25-45%	209.0	2022	99.5	0.049	33.4
			213.0	2021	100.2	0.050	33.6
			214.0	2021	99.7	0.049	33.5
		Average	212.3±0.54	2021.5±0.20	99.98±0.78	0.049±0.005	33.56±0.12
		45-65%	209.0	2111	121.5	0.058	40.8
			209.5	2111	121.8	0.058	40.9
			213.0	2109	120.6	0.057	40.5
		Average	210.5±0.49	2110.6±0.27	121.3±0.26	0.057±0.006	40.71±0.15

Table D2: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
30	7.5	25-45%	212.0	2329	135.50	0.058	45.5
			210.0	2328	136.80	0.059	45.9
			209.5	2327	135.40	0.058	45.5
		Average	210.5±0.38	2328.1±0.30	135.90±0.29	0.058±0.006	45.61±0.17
		45-65%	210.0	2526	154.90	0.061	52.0
			210.5	2526	155.70	0.062	52.3
			217.0	2526	153.20	0.061	51.4
		Average	212.5±0.65	2526.0±0.00	154.60±0.37	0.061±0.007	51.89±0.22
	8.0	25-45%	209.0	1622	59.20	0.036	19.9
			209.5	1623	58.40	0.036	19.6
			213.0	1620	57.75	0.036	19.4
		Average	210.5±0.49	1621.7±0.40	58.45±0.28	0.036±0.007	19.62±0.16
		45-65%	209.0	1792	75.40	0.042	25.3
			213.0	1791	76.10	0.042	25.5
			214.0	1793	75.90	0.042	25.5
		Average	212.3±0.54	1792.1±0.35	75.80±0.20	0.046±0.005	25.46±0.12

Table D3: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from mixture of lung and pancreas at an incubation time 45 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
45	6.5	25-45%	212.0	1612	69.50	0.043	23.32
			210.0	1610	68.50	0.042	22.99
			209.5	1610	68.16	0.042	22.88
		Average	210.5±0.38	1610.9±0.12	68.72±0.71	0.040±0.03	20.07±0.19
		45-65%	210.0	1718	69.50	0.040	23.3
			210.5	1719	68.50	0.040	23.0
			217.0	1715	93.10	0.054	31.3
		Average	212.5±0.65	1717.4±0.46	77.06±1.24	0.042±0.03	25.86±0.72
	7.0	25-45%	209.0	1731	83.60	0.048	28.1
			209.5	1733	83.40	0.048	28.0
			213.0	1727	81.91	0.048	27.5
		Average	210.5±0.49	1730.2±0.55	82.97±0.32	0.048±0.00	27.85±0.19
		45-65%	209.0	1792	93.82	0.052	31.5
			213.0	1793	92.80	0.052	31.2
			214.0	1791	91.75	0.051	30.8
		Average	212.3±0.54	1792.3±0.38	92.79±0.78	0.052±0.03	31.14±0.22

Table D3: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
45	7.5	25-45%	209.0	1863	98.6	0.053	33.1
			213.0	1863	97.5	0.052	32.7
			214.0	1861	97.0	0.052	32.6
		Average	212.3±0.54	1862.6±0.35	97.73±0.29	0.052±0.007	32.80±0.17
		45-65%	209.0	2036	114.8	0.059	38.5
			213.0	2037	113.8	0.057	38.2
			214.0	2035	114.0	0.058	38.3
		Average	212.3±0.54	2036.5±0.30	114.2±0.40	0.059±0.005	38.33±0.11
	8.0	25-45%	209.0	1566	50.5	0.032	17
			209.5	1565	50.2	0.032	16.9
			213.0	1567	48.4	0.031	16.2
		Average	210.5±0.49	1566.0±0.65	49.70±0.51	0.034±0.009	14.69±0.14
		45-65%	210.0	1585	69.5	0.046	23.3
			210.5	1585	67.8	0.045	22.8
			217.0	1581	67.6	0.047	22.7
		Average	212.5±0.65	1584.1±0.34	68.39±2.01	0.046±0.008	20.63±0.55

Table D4: Effects of pH and ammonium sulphate saturation levels on V, TP, TA, SA and Y of cystatin extracted from mixture of lung and pancreas at an incubation time 60 min.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
60	6.5	25-45%	212.0	1423	49.80	0.025	16.7
			210.0	1425	48.56	0.030	16.3
			209.5	1439	48.07	0.033	16.1
		Average	210.5±0.38	1429.0±2.98	48.81±2.70	0.030±0.005	16.38±0.75
		45-65%	210.0	1502	56.50	0.029	18.96
			210.5	1499	57.40	0.033	19.56
			217.0	1500	56.20	0.031	18.88
		Average	212.5±0.65	1500.8±0.25	56.72±0.25	0.031±0.007	19.03±0.15
	7.0	25-45%	209.0	1596	64.50	0.040	21.7
			209.5	1597	65.80	0.041	22.1
			213.0	1592	62.30	0.036	20.9
		Average	210.5±0.49	1595.4±0.51	64.21±0.44	0.035±0.011	21.55±0.26
		45-65%	209.0	1694	77.90	0.046	26.1
			213.0	1696	78.50	0.046	26.4
			214.0	1690	75.70	0.045	25.4
		Average	212.3±0.54	1693.4±0.57	77.38±0.40	0.043±0.009	25.97±0.23

Table D4: Continued.

Incubation Time (min)	pH	Saturation	V (mL)	TP (mg)	TA (Units)	SA (Units/mg)	Y (%)
60	7.5	25-45%	212.0	1629	76.51	0.041	25.7
			210.0	1630	76.31	0.045	25.6
			209.5	1625	75.84	0.043	25.5
		Average	210.5±0.38	1628.5±0.19	76.22±0.73	0.042±0.004	25.58±0.11
		45-65%	210.0	1782	76.51	0.042	25.68
			210.5	1781	76.31	0.042	25.61
			217.0	1782	76.81	0.045	24.51
		Average	212.5±0.65	1782.2±0.23	85.21±3.15	0.043±0.001	28.60±0.88
	8.0	25-45%	209.0	1335	37.90	0.020	12.7
			209.5	1337	40.50	0.021	13.6
			213.0	1334	33.68	0.020	11.3
		Average	210.5±0.49	1335.3±0.35	37.36±0.54	0.020±0.003	12.54±0.15
		45-65%	209.0	1406	49.80	0.035	16.7
			213.0	1409	46.50	0.027	15.6
			214.0	1408	48.30	0.030	16.2
		Average	212.3±0.54	1407.8±0.66	48.21±1.18	0.030±0.005	16.18±0.33

APPENDIX E

EFFECT OF pH ON INHIBITORY ACTIVITY OF CYSTATIN FROM LUNG, PANCREAS AND MIXTURE OF LUNG AND PANCREAS

Table E1: Effect of pH on inhibitory activity of cystatin from lung, pancreas and mixture of lung and pancreas (extracted at 45%-65% ammonium sulphate saturation, 30 min incubation time and pH-7.5)

pH	Inhibition (%) of cystatin		
	Lung sample	Pancreas sample	Lung and pancreas sample
2.5	0.0	0.0	0.0
	0.0	0.0	0.0
	0.0	0.0	0.0
Average	0.0±0.00	0.0±0.00	0.0±0.00
3.5	1.9	0.6	1.8
	1.8	0.8	0.5
	2.0	1.0	1.3
Average	1.9±0.05	0.8±0.12	1.2±0.37
4.5	11.2	6.5	8.6
	12.1	7.6	9.2
	11.2	8.1	9.5
Average	11.5±0.30	7.4±0.47	9.1±0.26
5.5	17.1	9.2	11.8
	17.7	8.6	13.5
	18.9	12.2	14.3
Average	17.9±0.52	10.0±1.11	13.2±0.74
6.5	49.5	16.5	27.9
	49.9	21.4	28.4
	47.0	20.3	31.6
Average	48.8±0.90	19.4±1.48	29.3±1.15

Replicates (n) =3

Table E1: Continued

pH	Inhibition (%) of cystatin		
	Lung sample	Pancreas sample	Lung and pancreas sample
7.5	52.5	24.4	38.2
	54.9	25.4	40.8
	58.0	21.6	40.1
Average	55.1±1.59	23.8±1.14	39.7±0.77
8.5	44.1	10.9	30.8
	44.6	18.5	32.3
	44.5	16.2	30.5
Average	44.4±0.15	15.2±2.25	31.2±0.55
9.5	39.2	11.7	24.6
	38.4	15.9	25.3
	38.2	9.6	19.4
Average	38.6±0.30	12.4±1.85	23.1±1.86
10.5	28.2	8.4	18.2
	29.6	12.5	21.6
	31.3	7.9	17.5
Average	29.7±0.89	9.6±1.45	19.1±1.26
11.5	0.0	0.0	0.0
	0.0	0.0	0.0
	0.0	0.0	0.0
Average	0.0±0.00	0.0±0.00	0.0±0.00

Replicates (n) =3

APPENDIX F

EFFECT OF TEMPERATURE ON INHIBITORY ACTIVITY OF CYSTATIN FROM LUNG, PANCREAS AND MIXTURE OF LUNG AND PANCREAS

Table F1: Effect of temperature on inhibitory activity of cystatin from lung, pancreas and mixture of lung and pancreas samples (extracted at 45%-65% ammonium sulphate saturation, 30min incubation time and pH-7.5)

Temperature	Inhibition (%) of cystatin		
	Lung sample	Pancreas sample	Lung and pancreas sample
30	44.7	12.5	37.1
	41.9	11.8	38.8
	40.9	14.4	36.3
Average	42.5±1.14	12.9±0.77	37.4±0.73
40	56.1	19.2	38.5
	55.8	19.8	39.8
	53.7	19.5	42
Average	55.2±0.75	19.5±0.17	40.1±1.02
50	56.1	25.1	44.2
	57.8	26.4	43.7
	56.5	25.9	43.5
	56.8±0.52	25.8±0.37	43.8±0.20
60	42.8	21.8	37.4
	44.5	20.3	39.2
	46.5	22.1	39.2
Average	44.6±1.06	21.4±0.55	38.6±0.60
70	39.6	16.2	30.8
	37.4	16.9	32.3
	38.5	17.0	31.1
Average	38.5±0.64	16.7±0.25	31.4±0.45

Replicates (n) =3

Table F1: Continued.

Temperature	Inhibition (%) of cystatin		
	Lung sample	Pancreas sample	Lung and pancreas sample
80	35.1	9.6	26.8
	33.8	11.5	28.1
	37.3	12.2	26.7
Average	35.4±1.02	11.1±0.77	27.2±0.46
90	29.6	8.5	19.5
	28.5	10.6	20.1
	26.8	9.1	19.2
Average	28.3±0.81	9.4±0.62	19.6±0.26

Replicates (n) =3